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(54) Title: STARCH PROCESS

(57) Abstract: The present invention relates to a process for enzymatic hydrolysis of granular starch into a soluble starch hydrolysate at a temperature below the initial gelatinization temperature of said granular starch.

STARCH PROCESS

FIELD OF THE INVENTION

The present invention relates to a one step process for hydrolysis of granular starch into a soluble starch hydrolysate at a temperature below the initial gelatinization temperature of said granular starch.

BACKGROUND OF THE INVENTION

A large number of processes have been described for converting starch to starch hydrolysates, such as maltose, glucose or specialty syrups, either for use as sweeteners or as precursors for other saccharides such as fructose. Glucose may also be fermented to ethanol or other fermentation products.

Starch is a high molecular-weight polymer consisting of chains of glucose units. It usually consists of about 80% amylopectin and 20% amylose. Amylopectin is a branched polysaccharide in which linear chains of alpha-1,4 D-glucose residues are joined by alpha-1,6 glucosidic linkages.

Amylose is a linear polysaccharide built up of D-glucopyranose units linked together by alpha-1,4 glucosidic linkages. In the case of converting starch into a soluble starch hydrolysate, the starch is depolymerized. The conventional depolymerization process consists of a gelatinization step and two consecutive process steps, namely a liquefaction process and a saccharification process.

Granular starch consists of microscopic granules, which are insoluble in water at room temperature. When an aqueous starch slurry is heated, the granules swell and eventually burst, dispersing the starch molecules into the solution. During this "gelatinization" process there is a dramatic increase in viscosity. As the solids level is 30-40% in a typical industrial process, the starch has to be thinned or "liquefied" so that it can be handled. This reduction in viscosity is today mostly obtained by enzymatic degradation. During the liquefaction step, the long-chained starch is degraded into smaller branched and linear units (maltodextrins) by an alpha-amylase. The liquefaction process is typically carried out at about 105-110°C for about 5 to 10 minutes followed by about 1-2 hours at about 95°C. The temperature is then lowered to 60°C, a glucoamylase or a beta-amylase and optionally a debranching enzyme, such as an isoamylase or a pullulanase are added, and the saccharification process proceeds for about 24 to 72 hours.

It will be apparent from the above discussion that the conventional starch conversion process is very energy consuming due to the different requirements in terms of temperature during the various steps. It is thus desirable to be able to select the enzymes used in the process so that the overall process can be performed without having to gelatinize the starch.

Such processes are the subject for the patents US4591560, US4727026 and US4009074 and EP0171218.

The present invention relates to a one-step process for converting granular starch into soluble starch hydrolysate at a temperature below initial gelatinization temperature of the starch.

SUMMARY OF THE INVENTION

In a first aspect the invention provides a one step process for producing a soluble starch hydrolysate, the process comprising subjecting a aqueous granular starch slurry at a temperature below the initial gelatinization temperature of said granular starch to the simultaneous action of the following enzyme activities, a first enzyme which is a member of the Glycoside Hydrolase Family 13, has alpha-1.4-glucosidic hydrolysis activity and comprises a Carbohydrate-Binding Module of Family 20, and a second enzyme which is a fungal alpha-amylase (EC 3.2.1.1), a beta-amylase (E.C. 3.2.1.2), or an glucoamylase (E.C.3.2.1.3).

In a second aspect the invention provides a process for production of high fructose starch-based syrup (HFSS), the process comprising producing a soluble starch hydrolysate by the process of the first aspect of the invention, and further comprising a step for conversion of the soluble starch hydrolysate into a of high fructose starch-based syrup (HFSS).

In a third aspect the invention provides a process for production of fuel or potable ethanol; comprising producing a soluble starch hydrolysate by the process of the first aspect of the invention, and further comprising a step for fermentation of the soluble starch hydrolysate into ethanol, wherein the fermentation step is carried out simultaneously or separately/sequential to the hydrolysis of the granular starch.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

The term "granular starch" is understood as raw uncooked starch, i.e. starch that has not been subjected to a gelatinization. Starch is formed in plants as tiny granules insoluble in water. These granules are preserved in starches at temperatures below the initial gelatinization temperature. When put in cold water, the grains may absorb a small amount of the liquid. Up to 50°C to 70°C the swelling is reversible, the degree of reversibility being dependent upon the particular starch. With higher temperatures an irreversible swelling called gelatinization begins.

The term "initial gelatinization temperature" is understood as the lowest temperature at which gelatinization of the starch commences. Starch begins to gelatinize between 60°C and 70°C, the exact temperature dependent on the specific starch. The initial gelatinization

temperature depends on the source of the starch to be processed. The initial gelatinization temperature for wheat starch is approximately 52°C, for potato starch approximately 56°C, and for corn starch approximately 62°C. However, the quality of the starch initial may vary according to the particular variety of the plant species as well as with the growth conditions and therefore initial gelatinization temperature should be determined for each individual starch lot.

The term "soluble starch hydrolysate" is understood as the soluble products of the processes of the invention and may comprise mono- di-, and oligosaccharides, such as glucose, maltose, maltodextrins, cyclodextrins and any mixture of these. Preferably at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97% or 98% of the dry solids of the granular starch is converted into a soluble starch hydrolysate.

The term "Speciality Syrups", is an in the art recognised term and is characterised according to DE and carbohydrate spectrum (See the article "New Speciality Glucose Syrups", p. 50+, in the textbook "Molecular Structure and Function of Food Carbohydrate", Edited by G.G. Birch and L.F. Green, Applied Science Publishers LTD., London). Typically Speciality Syrups have a DE in the range from 35 to 45.

The "Glycoside Hydrolase Family 13" is in the context of this invention defined as the group of hydrolases comprising a catalytic domain having a (beta/alpha)$_8$ or TIM barrel structure and acting on starch and related substrates through an alpha-retaining reacting mechanism (Koshland, 1953, Biol.Rev.Camp.Philos.Soc 28, 416-436).

The enzymes having "alpha-1.4-glucosidic hydrolysis activity" is in the context of this invention defined as comprising the group of enzymes which catalyze the hydrolysis and/or synthesis of alpha-1,4-glucosidic bonds as defined by Takata (Takata et al, 1992, J. Biol. Chem. 267, 18447-18452) and by Koshland (Koshland, 1953, Biol.Rev. Camp. Philos. Soc 28, 416-436).

The "Carbohydrate-Binding Module of Family 20" or a CBM-20 module is in the context of this invention defined as a sequence of approximately 100 amino acids having at least 45% homology to the Carbohydrate-Binding Module (CBM) of the polypeptide disclosed in figure 1 by Joergensen et al (1997) in Biotechnol. Lett. 19:1027-1031. The CBM comprises the last 102 amino acids of the polypeptide, i.e. the subsequence from amino acid 582 to amino acid 683.

Enzymes which; (a) are members of the Glycoside Hydrolase Family 13; (b) have alpha-1.4-glucosidic hydrolysis activity and (c) comprise a Carbohydrate-Binding Module of Family 20, and are specifically contemplated for this invention comprise the enzymes classified as EC 2.4.1.19, the cyclodextrin glucanotransferases, and EC 3.2.1.133, the maltogenic alpha-amylases, and selected members of 3.2.1.1 the alpha-amylases, and 3.2.1.60, the maltotetraose-forming amylases.

The "hydrolysis activity" of CGTases and maltogenic alpha-amylases is determined by measuring the increase in reducing power during incubation with starch according to Wind, R.D. et al 1995 in Appl. Environ. Microbiol.61:1257-1265. Reducing sugar concentrations is measured with the dinitrosalisylic acid method according to Bernfield (Bernfield, P. 1955. Amylases alpha and beta. Methods Enzymol. 1:149-158), with a few modifications. Diluted enzyme is incubated for an appropriate period of time with 1% (wt/v) soluble starch (Paselli SA2 starch from Avebe, The Netherlands or alternatively soluble starch from Merck) in a 10 mM sodium citrate (pH 5.9) buffer at 60°C. One unit of hydrolysis activity is defined as the amount of enzyme producing 1 micro mol of maltose per minute under standard conditions.

The polypeptide "homology" referred to in this disclosure is understood as the degree of identity between two sequences indicating a derivation of the first sequence from the second. The homology may suitably be determined by means of computer programs known in the art such as GAP provided in the GCG program package (Program Manual for the Wisconsin Package, Version 8, August 1994, Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA 53711) (Needleman, S.B. and Wunsch, C.D., (1970), Journal of Molecular Biology, 48, 443-453. The following settings for polypeptide sequence comparison are used: GAP creation penalty of 3.0 and GAP extension penalty of 0.1.

Cyclodextrin glucanotransferases (CGTases)

A particular enzyme to be used as a first enzyme in the processes of the invention may be a cyclomaltodextrin glucanotransferase (E.C. 2.4.1.19). Cyclomaltodextrin glucanotransferase, also designated cyclodextrin glucanotransferase or cyclodextrin glycosyltransferase, in the following termed CGTase, catalyses the conversion of starch and similar substrates into cyclomaltodextrins via an intramolecular transglycosylation reaction, thereby forming cyclomaltodextrins of various sizes. Most CGTases have both transglycosylation activity and starch-degrading activity. Contemplated CGTases are preferably of microbial origin, and most preferably of bacterial origin. Specifically contemplated CGTases include the CGTases having 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85% or even 90% homology to the sequence shown as amino acids 1 to 679 of SEQ ID NO:2 in WO02/06508, the CGTases having 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85% or even 90% homology to the amino acid sequence of the polypeptide disclosed in Joergensen et al, 1997 in figure 1 in Biotechnol. Lett. 19:1027-1031, and the CGTases described in US5278059 and US5545587. Preferably the CGTase to be applied as a first enzyme of the process has a hydrolysis activity of at least 3.5, preferably at least 4, 4.5, 5, 6, 7, 8, 9,10, 11, 12,13, 14, 15, 16, 17, 18, 19, 20, 21, 22, or most preferably at least 23 micro mol per min/mg. CGTases may be added in amounts of 0.01-100.0 NU/g DS, preferably from 0.2-50.0 NU/g DS, preferably 10.0-20.0 NU/g DS.

Maltogenic alpha-amylase

Another particular enzyme to be used as a first enzyme in the processes of the invention is a maltogenic alpha-amylase (E.C. 3.2.1.133). Maltogenic alpha-amylases (glucan 1,4-alpha-maltohydrolase) are able to hydrolyse amylose and amylopectin to maltose in the alpha-configuration. Furthermore, a maltogenic alpha-amylase is able to hydrolyse maltotriose as well as cyclodextrins. Specifically contemplated maltogenic alpha-amylases may be derived from *Bacillus* sp., preferably from *Bacillus stearothermophilus*, most preferably from *Bacillus stearothermophilus* C599 such as the one described in EP120.693. This particular maltogenic alpha-amylase has the amino acid sequence shown as amino acids 1-686 of SEQ ID NO:1 in US6162628. A preferred maltogenic alpha-amylase has an amino acid sequence having at least 70% identity to amino acids 1-686 of SEQ ID NO:1 in US6162628, preferably at least 80%, at least 85%, at least 90%, at least 92%, at least 95%, at least 96%, at least 97%, at least 98%, or particularly at least 99%. Most preferred variants of the maltogenic alpha-amylase comprise the variants disclosed in WO99/43794.

The maltogenic alpha-amylase having the amino acid sequence shown as amino acids 1-686 of SEQ ID NO:1 in US6162628 has a hydrolysis activity of 714. Preferably the maltogenic alpha-amylase to be applied as a first enzyme of the process has a hydrolysis activity of at least 3.5, preferably at least 4, 5, 6, 7, 8, 9,10, 11, 12,13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 100, 200, 300, 400, 500, 600, or most preferably at least 700 micro mol per min/mg.

Maltogenic alpha-amylases may be added in amounts of 0.01-40.0 MANU/g DS, preferably from 0.02-10 MANU/g DS, preferably 0.05-5.0 MANU/g DS.

Fungal alpha-amylase

A particular enzyme to be used as a second enzyme in the processes of the invention is a fungal alpha-amylase (EC 3.2.1.1), such as a fungamyl-like alpha-amylase. In the present disclosure, the term "fungamyl-like alpha-amylase" indicates an alpha-amylase which exhibits a high homology, i.e. more than 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85% or even 90% homology to the amino acid sequence shown in SEQ ID No. 10 in WO96/23874. Fungal alpha-amylases may be added in an amount of 0.001-1.0 AFAU/g DS, preferably from 0.002-0.5 AFAU/g DS, preferably 0.02-0.1 AFAU/g DS.

Beta-amylase

Another particular enzyme to be used as a second enzyme in the processes of the invention may be a beta-amylase (E.C 3.2.1.2). Beta-amylase is the name traditionally given to exo-acting maltogenic amylases, which catalyze the hydrolysis of 1,4-alpha-glucosidic linkages in amylose, amylopectin and related glucose polymers.

Beta-amylases have been isolated from various plants and microorganisms (W.M. Fogarty and C.T. Kelly, Progress in Industrial Microbiology, vol. 15, pp. 112-115, 1979). These

beta-amylases are characterized by having optimum temperatures in the range from 40°C to 65°C and optimum pH in the range from 4.5 to 7.0. Contemplated beta-amylase include the beta-amylase from barley Spezyme® BBA 1500, Spezyme® DBA and Optimalt™ ME, Optimalt™ BBA from Genencor int as well as Novozym™ WBA from Novozymes A/S.

Glucoamylase

A further particular enzyme to be used as a second enzyme in the processes of the invention may also be a glucoamylase (E.C.3.2.1.3) derived from a microorganism or a plant. Preferred is glucoamylases of fungal or bacterial origin selected from the group consisting of *Aspergillus* glucoamylases, in particular *A. niger* G1 or G2 glucoamylase (Boel et al. (1984), EMBO J. 3 (5), p. 1097-1102), or variants thereof, such as disclosed in WO92/00381 and WO00/04136; the *A. awamori* glucoamylase (WO84/02921), A. oryzae (Agric. Biol. Chem. (1991), 55 (4), p. 941-949), or variants or fragments thereof.

Other contemplated *Aspergillus* glucoamylase variants include variants to enhance the thermal stability: G137A and G139A (Chen et al. (1996), *Prot. Engng.* 9, 499-505); D257E and D293E/Q (Chen et al. (1995), *Prot. Engng.* 8, 575-582); N182 (Chen et al. (1994), *Biochem. J.* 301, 275-281); disulphide bonds, A246C (Fierobe et al. (1996), *Biochemistry*, 35, 8698-8704; and introduction of Pro residues in position A435 and S436 (Li et al. (1997), Protein *Engng.* 10, 1199-1204. Furthermore Clark Ford presented a paper on Oct 17, 1997, ENZYME ENGINEERING 14, Beijing/China Oct 12-17, 97, Abstract book p. 0-61. The abstract suggests mutations in positions G137A, N20C/A27C, and S30P in an *Aspergillus awamori* glucoamylase to improve the thermal stability. Other contemplated glucoamylases include *Talaromyces* glucoamylases, in particular derived from *Talaromyces emersonii* (WO99/28448), *Talaromyces leycettanus* (US patent no. Re.32,153), *Talaromyces duponti*, *Talaromyces thermophilus* (US patent no. 4,587,215). Bacterial glucoamylases contemplated include glucoamylases from the genus *Clostridium*, in particular *C. thermoamylolyticum* (EP135,138), and *C. thermohydrosulfuricum* (WO86/01831). Preferred glucoamylases include the glucoamylases derived from *Aspergillus oryzae*, such as a glucoamylase having 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85% or even 90% homology to the amino acid sequence shown in SEQ ID NO:2 in WO00/04136. Also contemplated are the commercial products AMG 200L; AMG 300 L; SAN™ SUPER and AMG™ E (from Novozymes); OPTIDEX™ 300 (from Genencor Int.); AMIGASE™ and AMIGASE™ PLUS (from DSM); G-ZYME™ G900 (from Enzyme Bio-Systems); G-ZYME™ G990 ZR (*A. niger* glucoamylase and low protease content).

Glucoamylases may be added in an amount of 0.02-2.0 AGU/g DS, preferably 0.1-1.0 AGU/g DS, such as 0.2 AGU/g DS.

Additional enzymes.

The processes of the invention may also be carried out in the presence of a third enzyme. A particular third enzyme may be a *Bacillus* alpha-amylase (often referred to as

"Termamyl-like alpha-amylases"). Well-known Termamyl-like alpha-amylases include alpha-amylase derived from a strain of *B. licheniformis* (commercially available as Termamyl), *B. amyloliquefaciens,* and *B. stearothermophilus* alpha-amylase. Other Termamyl-like alpha-amylases include alpha-amylase derived from a strain of the *Bacillus* sp. NCIB 12289, NCIB 12512, NCIB 12513 or DSM 9375, all of which are described in detail in WO95/26397, and the alpha-amylase described by Tsukamoto et al., Biochemical and Biophysical Research Communications, 151 (1988), pp. 25-31. In the context of the present invention a Termamyl-like alpha-amylase is an alpha-amylase as defined in WO99/19467 on page 3, line 18 to page 6, line 27. Contemplated variants and hybrids are described in WO96/23874, WO97/41213, and WO99/19467. Specifically contemplated is a recombinant *B.stearothermophilus* alpha-amylase variant with the mutations: I181* + G182* + N193F. *Bacillus* alpha-amylases may be added in effective amounts well known to the person skilled in the art.

Another particular third enzyme of the process may be a debranching enzyme, such as an isoamylase (E.C. 3.2.1.68) or a pullulanases (E.C. 3.2.1.41). Isoamylase hydrolyses alpha-1,6-D-glucosidic branch linkages in amylopectin and beta-limit dextrins and can be distinguished from pullulanases by the inability of isoamylase to attack pullulan, and by the limited action on alpha-limit dextrins. Debranching enzyme may be added in effective amounts well known to the person skilled in the art.

Embodiments of the invention

The starch slurry to be subjected to the processes of the invention may have 20-55% dry solids granular starch, preferably 25-40% dry solids granular starch, more preferably 30-35% dry solids granular starch.

After being subjected to the process of the first aspect of the invention at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or preferably 99% of the dry solids of the granular starch is converted into a soluble starch hydrolysate.

According to the invention the processes of the first and second aspect is conducted at a temperature below the initial gelatinization temperature. Preferably the temperature at which the processes are conducted is at least 30°C, 31°C, 32°C, 33°C, 34°C, 35°C, 36°C, 37°C, 38°C, 39°C, 40°C, 41°C, 42°C, 43°C, 44°C, 45°C, 46°C, 47°C, 48°C, 49°C, 50°C, 51°C, 52°C, 53°C, 54°C, 55°C, 56°C, 57°C, 58°C, 59°C, or preferably at least 60°C.

The pH at which the process of the first aspect of the invention is conducted may in be in the range of 3.0 to 7.0, preferably from 3.5 to 6.0, or more preferably from 4.0-5.0.

The exact composition of the products of the process of the first aspect of the invention, the soluble starch hydrolysate, depends on the combination of enzymes applied as well as the type of granular starch processed. Preferably the soluble hydrolysate is maltose with a purity of at least 85%, 90%, 95.0%, 95.5%, 96.0%, 96.5%, 97.0%, 97.5%, 98.0%, 98.5, 99.0% or 99.5%. Even more preferably the soluble starch hydrolysate is glucose, and most

preferably the starch hydrolysate has a DX (glucose percent of total solubilised dry solids) of at least 94.5%, 95.0%, 95.5%, 96.0%, 96.5%, 97.0%, 97.5%, 98.0%, 98.5, 99.0% or 99.5%. Equally contemplated, however, is the process wherein the product of the process of the invention, the soluble starch hydrolysate, is a speciality syrup, such as a speciality syrup containing a mixture of glucose, maltose, DP3 and DPn for use in the manufacture of ice creams, cakes, candies, canned fruit.

The granular starch to be processed in the processes of the invention may in particular be obtained from tubers, roots, stems, legumes, cereals or whole grain. More specifically the granular starch may be obtained from corns, cobs, wheat, barley, rye, milo, sago, cassava, taploca, sorghum, rice, peas, bean, banana or potatoes. Specially contemplated are both waxy and non-waxy types of corn and barley. The granular starch to be processed may be a highly refined starch quality, preferably more than 90%, 95%, 97% or 99.5 % pure or it may be a more crude starch containing material comprising milled whole grain including non-starch fractions such as germ residues and fibres. The raw material, such as whole grain, is milled in order to open up the structure and allowing for further processing. Two milling processes are preferred according to the invention: wet and dry milling. In dry milling the whole kernel is milled and used. Wet milling gives a good separation of germ and meal (starch granules and protein) and is with a few exceptions applied at locations where the starch hydrolysate is used in production of syrups. Both dry and wet milling is well known in the art of starch processing and are equally contemplated for the processes of the invention.The process of the first aspect of the invention may be conducted in an ultrafiltration system where the retentate is held under recirculation in presence of enzymes, raw starch and water and where the permeate is the soluble starch hydrolysate. Equally contemplated is the process conducted in a continuous membrane reactor with ultrafiltration membranes and where the retentate is held under recirculation in presence of enzymes, raw starch and water and where the permeate is the soluble starch hydrolysate. Also contemplated is the process conducted in a continuous membrane reactor with microfiltration membranes and where the retentate is held under recirculation in presence of enzymes, raw starch and water and where the permeate is the soluble starch hydrolysate.

In the process of the second aspect of the invention the soluble starch hydrolysate of the process of the first aspect of the invention is subjected to conversion into high fructose starch-based syrup (HFSS), such as high fructose corn syrup (HFCS). This conversion is preferably achieved using a glucose isomerase, and more preferably by an immobilized glucose isomerase supported on a solid support. Contemplated isomerases comprises the commercial products Sweetzyme™ IT from Novozymes A/S; G -zyme™ IMGI and G-zyme™ G993, Ketomax™ and G-zyme™ G993 from Rhodia, G-zyme™ G993 liquid and GenSweet™ IGI from Genemcor Int.

In the process of the third aspect of the invention the soluble starch hydrolysate of the process of the first aspect of the invention is used for production of fuel or potable ethanol. In the process of the third aspect the fermentation may be carried out simultaneously or separately/sequential to the hydrolysis of the granular starch slurry. When the fermentation is performed simultaneous to the hydrolysis the temperature is preferably between 30°C and 35°C, and more preferably between 31°C and 34°C. The process of the third aspect of the invention may be conducted in an ultrafiltration system where the retentate is held under recirculation in presence of enzymes, raw starch, yeast, yeast nutrients and water and where the permeate is an ethanol containing liquid. Equally contemplated is the process conducted in a continuous membrane reactor with ultrafiltration membranes and where the retentate is held under recirculation in presence of enzymes, raw starch, yeast, yeast nutrients and water and where the permeate is an ethanol containing liquid.

MATERIALS AND METHODS

Alpha-amylase activity (KNU)

The amylolytic activity may be determined using potato starch as substrate. This method is based on the break-down of modified potato starch by the enzyme, and the reaction is followed by mixing samples of the starch/enzyme solution with an iodine solution. Initially, a blackish-blue colour is formed, but during the break-down of the starch the blue colour gets weaker and gradually turns into a reddish-brown, which is compared to a coloured glass standard.

One Kilo Novo alpha amylase Unit (KNU) is defined as the amount of enzyme which, under standard conditions (i.e. at 37°C +/- 0.05; 0.0003 M Ca^{2+}; and pH 5.6) dextrinizes 5.26 g starch dry substance Merck Amylum solubile.

A folder AF 9/6 describing this analytical method in more detail is available upon request to Novozymes A/S, Denmark, which folder is hereby included by reference.

CGTase activity (KNU)

The CGTase alpha-amylase activity is determined by a method employing Phadebas® tablets as substrate. Phadebas tablets (Phadebas® Amylase Test, supplied by Pharmacia Diagnostic) contain a cross-linked insoluble blue-colored starch polymer, which has been mixed with bovine serum albumin and a buffer substance.

For every single measurement one tablet is suspended in a tube containing 5 ml 50 mM Britton-Robinson buffer (50 mM acetic acid, 50 mM phosphoric acid, 50 mM boric acid, 0.1 mM CaCl2, pH adjusted to the value of interest with NaOH). The test is performed in a water bath at the temperature of interest. The alpha-amylase to be tested is diluted in x ml of 50 mM Britton-Robinson buffer. 1 ml of this alpha-amylase solution is added to the 5 ml 50

mM Britton-Robinson buffer. The starch is hydrolyzed by the alpha-amylase giving soluble blue fragments. The absorbance of the resulting blue solution, measured spectrophotometrically at 620 nm, is a function of the alpha-amylase activity.

It is important that the measured 620 nm absorbance after 10 or 15 minutes of incubation (testing time) is in the range of 0.2 to 2.0 absorbance units at 620 nm. In this absorbance range there is linearity between activity and absorbance (Lambert-Beer law). The dilution of the enzyme must therefore be adjusted to fit this criterion. Under a specified set of conditions (temperature, pH, reaction time, buffer conditions) 1 mg of a given alpha-amylase will hydrolyze a certain amount of substrate and a blue colour will be produced. The colour intensity is measured at 620 nm. The measured absorbance is directly proportional to the specific activity (activity/mg of pure alpha-amylase protein) of the alpha-amylase in question under the given set of conditions.

A folder EAL-SM-0351 describing this analytical method in more detail is available upon request to Novozymes A/S, Denmark, which folder is hereby included by reference

Maltogenic alpha-amylase activity (MANU)

One Maltogenic Amylase Novo Unit (MANU) is defined as the amount of enzyme which under standard will cleave one micro mol maltotriose per minute. The standard conditions are 10 mg/ml maltotriose, 37°C, pH 5.0, and 30 minutes reaction time. The formed glucose is converted by glucose dehydrogenase (GlucDH, Merck) to gluconolactone under formation of NADH, which is determined spectophotometrically at 340 nm. A folder (EAL-SM-0203.01) describing this analytical method in more detail is available on request from Novozymes A/S, Denmark, which folder is hereby included by reference.

Glucoamylase activity (AGU)

The Novo Glucoamylase Unit (AGU) is defined as the amount of enzyme, which hydrolyzes 1 micromole maltose per minute at 37°C and pH 4.3.

The activity is determined as AGU/ml by a method modified after (AEL-SM-0131, available on request from Novozymes) using the Glucose GOD-Perid kit from Boehringer Mannheim, 124036. Standard: AMG-standard, batch 7-1195, 195 AGU/ml. 375 microL substrate (1% maltose in 50 mM Sodium acetate, pH 4.3) is incubated 5 minutes at 37°C. 25 microL enzyme diluted in sodium acetate is added. The reaction is stopped after 10 minutes by adding 100 microL 0.25 M NaOH. 20 microL is transferred to a 96 well microtitre plate and 200 microL GOD-Perid solution (124036, Boehringer Mannheim) is added. After 30 minutes at room temperature, the absorbance is measured at 650 nm and the activity calculated in AGU/ml from the AMG-standard. A folder (AEL-SM-0131) describing this analytical method in more detail is available on request from Novozymes A/S, Denmark, which folder is hereby included by reference.

Fungal alpha-amylase activity (FAU)

The alpha-amylase activity is measured in FAU (Fungal Alpha-Amylase Units). One (1) FAU is the amount of enzyme which under standard conditions (i.e. at 37°C and pH 4.7) breaks down 5260 mg solid starch (Amylum solubile, Merck) per hour. A folder AF 9.1/3, describing this FAU assay in more details, is available upon request from Novozymes A/S, Denmark, which folder is hereby included by reference.

Acid alpha-amylase activity (AFAU)

Acid alpha-amylase activity is measured in AFAU (Acid Fungal Alpha-amylase Units), which are determined relative to an enzyme standard.

The standard used is AMG 300 L (from Novozymes A/S, glucoamylase wildtype Aspergillus niger G1, also disclosed in Boel et al. (1984), EMBO J. 3 (5), p. 1097-1102 and in WO92/00381). The neutral alpha-amylase in this AMG falls after storage at room temperature for 3 weeks from approx. 1 FAU/mL to below 0.05 FAU/mL.

The acid alpha-amylase activity in this AMG standard is determined in accordance with the following description. In this method 1 AFAU is defined as the amount of enzyme, which degrades 5.26 mg starch dry solids per hour under standard conditions.

Iodine forms a blue complex with starch but not with its degradation products. The intensity of colour is therefore directly proportional to the concentration of starch. Amylase activity is determined using reverse colorimetry as a reduction in the concentration of starch under specified analytic conditions.

	Alpha-amylase	
Starch + Iodine	⟶	Dextrins + Oligosaccharides
	40°C, pH 2.5	
Blue/violet	t=23 sec.	Decoloration

Standard conditions/reaction conditions: (per minute)

Substrate:	starch, approx. 0.17 g/L
Buffer:	Citate, approx. 0.03 M
Iodine (I2):	0.03 g/L
CaCl2:	1.85 mM
pH:	2.50 - 0.05
Incubation temperature:	40°C
Reaction time:	23 seconds
Wavelength:	lambda=590nm
Enzyme concentration:	0.025 AFAU/mL
Enzyme working range:	0.01-0.04 AFAU/mL

If further details are preferred these can be found in EB-SM-0259.02/01 available on request from Novozymes A/S, and incorporated by reference.

Beta-amylase activity (DP°)

The activity of SPEZYME® BBA 1500 is expressed in Degree of Diastatic Power (DP°). It is the amount of enzyme contained in 0.1 ml of a 5% solution of the sample enzyme preparation that will produce sufficient reducing sugars to reduce 5 ml of Fehling's solution when the sample is incubated with 100 ml of substrate for 1 hour at 20°C.

Pullulanase activity (New Pullulanase Unit Novo (NPUN)

Pullulanase activity may be determined relative to a pullulan substrate. Pullulan is a linear D-glucose polymer consisting essentially of maltotriosyl units joined by 1,6-alpha-links. Endo-pullulanases hydrolyze the 1,6-alpha-links at random, releasing maltotriose, 6^3-alpha-maltotriosyl-maltotriose, 6^3-alpha-(6^3-alpha-maltotriosyl-maltotriosyl)-maltotriose.

One new Pullulanase Unit Novo (NPUN) is a unit of endo-pullulanase activity and is measured relative to a Novozymes A/S Promozyme D standard. Standard conditions are 30 minutes reaction time at 40°C and pH 4.5; and with 0.7% pullulan as substrate. The amount of red substrate degradation product is measured spectrophotometrically at 510 nm and is proportional to the endo-pullulanase activity in the sample. A folder (EB-SM.0420.02/01) describing this analytical method in more detail is available upon request to Novozymes A/S, Denmark, which folder is hereby included by reference.

Under the standard conditions one NPUN is approximately equal to the amount of enzyme which liberates reducing carbohydrate with a reducing power equivalent to 2.86 micromole glucose per minute.

Determination of CGTase hydrolysis activity

The CGTase hydrolysis activity was determined by measuring the increase in reducing power during incubation with Paselli SA2 starch (from Avebe, The Netherlands) as described by Wind et al. 1995 in Appl. Environ. Microbiol. 61: 1257-1265.

Determination of sugar profile and solubilised dry solids

The sugar composition of the starch hydrolysates was determined by HPLC and glucose yield was subsequently calculated as DX. °BRIX, solubilised (soluble) dry solids of the starch hydrolysates were determined by refractive index measurement.

Materials

The following enzyme activities were used. A maltogenic alpha-amylase with the amino acid sequence shown in SEQ ID No: 1 in WO9/943794. A glucoamylase derived from *Aspergillus oryzae* having the amino acid sequence shown in WO00/04136 as SEQ ID No: 2 or one of the disclosed variants. An acid fungal alpha-amylase derived from *Aspergillus niger*. A *Bacillus* alpha-amylase which is a recombinant *B.stearothermophilus* variant with the mutations: I181*+ G182*+N193F. A fungal alpha-amylase derived from *Aspergillus oryzae*. A CGTase N with the sequence shown herein as SEQ ID NO 1. A CGTase O with the sequence shown herein as SEQ ID NO 2. A CGTase T with the amino acid sequence disclosed in figure 1 in Joergensen et al (1997) in Biotechnol. Lett. 19:1027-1031 and shown herein as SEQ ID NO 3. A CGTase A having the sequence shown herein as SEQ ID NO 4.

Common corn starch (C x PHARM 03406) was obtained from Cerestar.

Example 1

This example illustrates the conversion of granular starch into glucose using CGTase T and a glucoamylase and an acid fungal amylase. A slurry with 33% dry solids (DS) granular starch was prepared by adding 247.5 g of common corn starch under stirring to 502.5 ml of water. The pH was adjusted with HCl to 4.5. The granular starch slurry was distributed to 100 ml blue cap flasks with 75 g in each flask. The flasks were incubated with magnetic stirring in a 60°C water bath. At zero hours the enzyme activities given in table 1 were dosed to the flasks. Samples were withdrawn after 24, 48, 72, and 96 hours.

Table 1. The enzyme activity levels used were:

CGTase T KNU/kg DS	Glucoamylase AGU/kg DS	Acid fungal alpha-amylase AFAU/kg DS
12.5	200	50
25.0	200	50
100.0	200	50

Total dry solids starch was determined using the following method. The starch was completely hydrolyzed by adding an excess amount of alpha-amylase (300 KNU/Kg dry solids) and subsequently placing the sample in an oil bath at 95 °C for 45 minutes. After filtration through a 0.22 microM filter the dry solids was measured by refractive index measurement.

Soluble dry solids in the starch hydrolysate were determined on samples after filtering through a 0.22 microM filter. Soluble dry solids were determined by refractive index measurement and the sugar profile was determined by HPLC. The amount of glucose was calculated as DX. The results are shown in table 2 and 3.

Table 2. Soluble dry solids as percentage of total dry substance at the three CGTase activity levels.

KNU/kg DS	24 hours	48 hours	72 hours	96 hours
12.5	68	82	89	94
25.0	76	89	93	97
100.0	83	96	98	99

Table 3. The DX of the soluble hydrolysate at the three CGTase activity levels.

KNU/kg DS	24 hours	48 hours	72 hours	96 hours
12.5	92.6	94.5	95.1	95.3
25.0	92.4	94.8	95.4	95.5
100.0	92.7	94.9	95.4	95.4

Example 2

This example illustrates the conversion of granular starch into glucose using CGTase T, a glucoamylase, an acid fungal alpha-amylase and a Bacillus alpha-amylase.

Flasks with 33% DS granular starch were prepared and incubated as described in example 1. At zero hours the enzymes activities given in table 4 were dosed to the flask.

Table 4. The enzyme activity levels used were:

CGTase T KNU/kg DS	Glucoamylase AGU/kg DS	Acid fungal alpha-amylase AFAU/kg DS	*Bacillus* alpha-amylase KNU/kg DS
5.0	200	50	300

Samples were withdrawn after 24, 48, 72, and 96 hours and analyzed as described in example 1. The results are shown in table 4 and 5.

Table 5. Soluble dry solids as percentage of total dry substance.

24 hours	48 hours	72 hours	96 hours
82.8	93.0	96.3	98.7

Table 6. The DX of the soluble hydrolysate.

24 hours	48 hours	72 hours	96 hours
92.8	94.9	95.5	95.8

Example 3

This example illustrates the conversion of granular starch into glucose using a maltogenic alpha-amylase, a glucoamylase and an acid fungal alpha-amylase.

Flasks with 33% DS granular starch were prepared and incubated as described in example 1. At zero hours the enzyme activities given in table 6 were dosed to the flasks.

Table 6. The enzyme activity levels used were:

	Maltogenic alpha-amylase MANU/kg DS	Glucoamylase AGU/kg DS	Acid fungal alpha-amylase AFAU/kg DS
Flask 1	5000	200	50
Flask 2	20000	200	50

Samples were withdrawn after 24, 48, 72, and 96 hours and analyzed as described in example 1. The results are shown in table 7 and 8.

Table 7. Soluble dry solids as percentage of total dry substance at the two maltogenic alpha-amylase activity levels.

MANU/kg DS	24 hours	48 hours	72 hours	96 hours
5000	63.1	75	79.3	85.3
20000	67.0	77.9	82.7	88.1

Table 8. The DX of the soluble hydrolysate at the two maltogenic alpha-amylase activity levels.

MANU/kg DS	24 hours	48 hours	72 hours	96 hours
5000	95.2	95.4	95.3	95.5
20000	93.8	94.9	94.9	94.8

Example 4

This example illustrates the only partial conversion of granular starch into glucose using a glucoamylase and an acid fungal alpha-amylase.

Flasks with 33% DS granular starch were prepared and incubated as described in example 1. At zero hours the enzyme activities given in table 9 were dosed to the flasks. Samples were withdrawn after 24, 48, 72, and 96 hours. The samples were analyzed as described in examples 1. The results are shown in table 10 and 11.

Table 9. The enzyme activity level used were:

Glucoamylase AGU/kg DS	Acid fungal alpha-amylase AFAU/kg DS
200	50

Table 10. Soluble dry solids as percentage of total dry substance.

24 hours	48 hours	72 hours	96 hours
28.5	36.3	41.6	45.7

Table 11. DX of the soluble hydrolysate.

24 hours	48 hours	72 hours	96 hours
27.7	34.9	39.2	42.2

Example 5

This example illustrates the correlation between the hydrolysis activity of four different CGTases (CGTase A, CGTase N, CGTase O and CGTase T) versus the yield during conversion of granular starch into glucose syrup using a CGTase and a glucoamylase measured as soluble dry solids and development in DX.

Flasks with 33% DS granular starch were prepared and incubated as described in example 1. At zero hour the CGTases were all dosed at 100 KNU/kg DS in combination with glucoamylase at 200 AGU/kg DS. Samples were withdrawn at 48 hours and analyzed as described in examples 1. Results are presented in table 12.

Table 12. Hydrolysis activity (micro mol per min/mg protein), and soluble dry solids (DS) and DX after 48 hors

CGTase	Hydrolysis act.	Soluble DS	DX
CGTase N	0.27	37.4	35.1
CGTase A	0.38	49.9	46.7
CGTase O	1.62	60.9	57.1
CGTase T	4.59	97.9	91.2

Example 6

This example illustrates the process conducted in an ultrafiltration system where the retentate was held under recirculation in presence of enzymes, raw starch and water and where the permeate is the soluble starch hydrolysate. A slurry comprising 100 kg granular corn starch suspended in 233 L tap city water and CGTase T (12.5 KNU/kg starch), *Bacillus* alpha-amylase (300 KNU/kg starch) and glucoamylase (200 AGU/kg starch) was processed in a batch ultrafiltration system (type PCI) with a tubular membrane module (type PU 120). The slurry was stirred at 100 rpm, pH was adjusted to 4.5 using 170 mL of 30 % HCl, and the reaction temperature was set at 57°C.

Samples of permeate and retentate were analyzed for dry solids content and for sugar composition.

The correction factor for non soluble material is: q = (100-S%)/(100-°BRIX). The centrifugation index for sugar is: ciS% = °BRIX/S% (no correction). The theoretical yield of sugar (glucose) S_{yield} = ciS%*q*100/111*100 %. A correction has thus been done for 100 kg starch dry matter giving ca. 111 kg glucose dry matter as a result of the hydrolysis reaction.

A trial was made in a simple batch system using the same enzyme system as for the membrane trial. As the comparison in table 15 a and b shows the membrane system reached the maximal solubilisation of starch earlier.

Table 13. Dry solids content and sugar composition of retentate and permeate

Sample	Hours	Reactor volume, L	% DS	% DP1	% DP2	% DP3	% DP4
Reactor	3	207	16.1	75.3	10.3	2.6	11.5
Reactor	28	123	28.3	95.0	2.7	0.8	1.5
Reactor	53	123	31.4	95.2	3.4	0.5	0.9
Permeate	3	207	12.1	71.2	17.4	2.9	8.5
Permeate	28	123	21.8	94.9	2.9	0.8	1.3

Tabel 14. Dry solids distribution in retentate at 3, 28, 53 and 77 hours.

	3 hours	28 hours	53 hours	77 hours
Soluble DS	16	28	31	39
Total DS	38	37	42	45

Table 15 a. Theoretical yield of glucose versus time for the membrane system

Hours	% total DS in the reactor	°BRIX	q=(100-S%)/(100-°Brix)	cis%=°Brix/S%	Theoretical yield sis=cis*q*100/111%
0	27.0	2.2	0.75	0.08	5
24	35.9	27.3	0.88	0.76	73
48	41.2	30.0	0.84	0.73	89
72	41.2	33.1	0.88	0.80	98
94	41.2	34.8	0.90	0.85	103

Table 15 b. Theoretical yield of glucose versus time for a batch reactor system.

Hours	% total DS in the reactor	°BRIX	q=(100-S%)/(100-°Brix)	cis%=°Brix/S%	Theoretical yield sis=cis*q*100/111%
0	29.7	2.0	0.72	0.07	4.
24	29.7	25.6	0.95	0.86	74
48	29.7	28.8	0.99	0.97	86
72	29.7	29.8	1.00	1.00	91
94	29.7	29.8	1.00	1.00	91

The conclusion was that when substrate saturation was maintained during the saccharification in a membrane system the degree of solubilization was improved compared to a simple batch reactor system for cold saccharification of raw starch.

Example 7

This example illustrates a simultaneous cold liquefaction and saccharification process of the invention carried out in a continuous working microfiltration membrane reactor using a ceramic module.

A 200 L feed mixer tank was connected by a reactor feed pump to a 200 L reactor tank with temperature control. Using a pump with a capacity of 0-20 l/h the mixture from the reactor was recycled through a APV ceramic microfiltration module for separation of glucose. Pore size was 0.2 micro m and the membrane area was 0.2 m2.

The reactor worked for about 200 hours using a dosage of 100 KNU/kg DS CGT-ase T and 300 AGU/kg DS of glucoamylase. With an average holding time in the reactor of 35-45 hours the system operated at steady state for the full period producing a DP1= 93 % glucose syrup at a yield of close to 100 %.

The reactor tank was loaded with 60 kg of corn starch type Cerestar C x PHARM 03406 suspended in 140 L of tap city water of 58°C under stirring. Using the steam heated mantel the temperature was adjusted to 60°C. Using 30 % HCl the pH was lowered from 6.1 to 4.5. The pH was re-checked (pH=4.5) aft er 15 minutes. At zero hours, immediately before adding the enzymes, CGTase T (100 KNU/kg starch) and glucoamylase (300 AGU/kg starch), samples were taken for determination of % sludge volume after centrifugation at 3000 rpm for 3 min in a table centrifuge. Furthermore the °BRIX of the supernatant was measured using a refractometer. The course of the reaction was followed regularly by measurements of sludge volumes and °BRIX of the supernatants as described above.

The feed mixer tank was loaded with 186 L of cold tap city water and 80 kg corn starch type Cerestar C x PHARM 03406. The feed mixer was kept stirred gentle and pH was adjusted to 4.5 using 30 % HCl. The temperature was kept at 7-8°C using cooling water and the enzymes CGTase T (100 KNU/kg starch) and glucoamylase (300 AGU/kg starch) was added. The low temperature secured that no reaction took place.

The upstart of the reactor was continued until the °Brix-value after 30 hours had stabilized around 27. Then the microfiltration was initiated using a pressure drop of 0.15 Bar and maximal retentate flow to secure this pressure. The filtrate was recycled to the reactor tank the first 5.7 hours. Hereafter the filtrate was collected in a separate tank, and the volume was measured as a function of time. At this point of time the reactor feed pump was started and adjusted to a flow rate equivalent to the filtrate flux (L/min). By doing so the volume in the reactor tank was kept constant.

The feed of starch slurry was continued while samples were taken as described above. Furthermore samples of the filtrates were taken. Any decrease in the filtrate flux were compensated for by increasing the retentate flow whereby the filter cake on the membrane was disrupted. Thereby the pressure drop was increased too. Samples were taken as a function of time of the filtrate for HPLC and °BRIX as well as the volume collected was measured. Simultaneously samples were taken from the reactor for measuring of total DS, sludge, °Brix and HPLC for sugar composition.

The trial lasted 220 hours. At that point of time the pressure drop was increased to about 0.4 Bar.

Determination of filtrate flux (based on single determinations) and average filtrate flux values (integrated) as a function of the process time showed that the enzyme system consisting of a CGTase and a glucoamylase alone maintained and secured a stable flux over a long processing time. This underlines the industrial potential advantages of this stable system.

The results and a mass balance are presented in tables 16-18.

Table 16. Analyses of collected filtrates.

Date and time	Hours from start	Collected filtrate, L	% DS w/w	Density, kg/L	Mass of DS, kg	Average flux, mL/min
13/03/02 16:05	30*	-	-	-	-	-
14/03/02 16:50	55	142	25.8	1.12	41.1	95.6
16/03/02 16:00	102	187	25.6	1.12	53.7	66.1
18/03/02 13:02	147	200	28.7	1.14	65.2	74.0
19/03/02 16:45	174	100	29.6	1.14	33.8	60.1
Total collected		629.0	27.3	1.13	193.7	-

*Start of continuous feeding to the reactor

Table 17. Composition of the syrup produced

% DP1	%DP2	% DP3	%DP4
93	5	1	2

Table 18. Mass balance for the trial of example 7

	Mass, kg	% DS	Mass of DS, kg	% Yield of DS*
Upstart of reactor				
Starch	60	90	54	25
Water	140	0	0	
Reactor start	200	27.0	54	25
Continuous production				
Starch consumption (t=28.75 h to t=174.5 h)	235.48	90	212	100
Water consumption (t=28.75 h to t=174.5 h)	548.12	0	0	
Substrate consumption	783.6	27.0	212	100
Syrup production	629.0	27.3	172	81
Reactor at end				
Total content	200	35	70	33
Unconverted starch	18	50	9	4
Mud, L	18	50	9	4
Glucose syrup	164	30	49	23

*basis substrate consumption at continuous production.

Compared to a batch trial carried out in a simple tank with stirring a significant reduction of the reaction time was obtained using the setup for hydrolysis of granular starch described above. As no viscosity problems were encountered with 30% DS it is considered feasible to increase the DS to 40%, or even as high as 45% and still maintain a smooth operation.

Example 8

This example compares a process of the invention and a conventional process for production of fuel ethanol or potable alcohol from raw starch in the form of dry milled corn, Yellow Dent No. 2.

A slurry of 30 % DS of dry milled corn was prepared in tap water in 250 ml blue cap flasks and the raw corn starch exposed to simultaneous cold liquefaction and pre-saccharification by a process of the invention. The slurry was heated to 60 °C in a water bath under magnet stirring, pH adjusted to 4.5 using 30 % HCl and CGTase T (75 KNU/kg DS) and glucoamylase (500 AGU/kg DS) added. After 48 hours the flask was cooled in the water bath to 32 °C.

A slurry of 30 % DS dry milled corn was pre-liquefied in a conventional continuous process consisting of a pre-liquefaction vessel, a jet-cooker, a flash, and a post liquefaction vessel. *Bacillus* alpha-amylase was added during the pre-liquefaction at 70-90°C (10 KNU/kg DS) and again during the post liquefaction at ca. 85-90°C (20 KNU/kg DS). The jet-cooking was carried out at 115-120°C. Pre-saccharification was performed under magnet stirring by

heating the mash in blue cap flasks to 60 °C in a water bath. After pH adjustment to 4.5 using 30 % HCl glucoamylase was added in a dosage equivalent to 500 AGU/kg DS. After 48 hours the flask was cooled in the water bath to 32 °C.

Fermentations were made directly in the blue cap flasks fitted with yeast locks filled with soybean oil. Bakers yeast (*Saccharomyces cerevisiae*) was added in an amount equivalent to 10 millions/mL of viable yeast cells and yeast nutrition in the form of 0.25 % urea was added to each flask. Each treatment was performed in 3 replicates.

The fermentation was monitored by the CO_2 loss as determined by weighing the flasks at regular intervals. L EtOH/100 kg grain dry matter (DS) was then calculated using the following formula:

$$\text{L EtOH/100 kg mash dry matter} = \frac{\text{Weight loss (g)} \times 1.045}{0.79\,(\text{g/mL}) \times 250 \times 30\%\ \text{dry matter}} \times 100$$

The mash contained 30 % w/w grain dry matter. 0.79 g/mL is the density of ethanol.

Tables 19 and 20 shows the obtained fermentation results for the replicates including the results of statistical calculation of the two types of pretreated raw materials (missing results estimated by interpolation).

Table 19. Fermentation result for the process of the invention using CGTase T (75 KNU/kg DS) and glucoamylase (500 AGU/kg DS).

Hour	L EtOH/100 kg grain	STDEV
0	-	-
25.5	28,3	0.9
48	35,4	0.6
69	37,1	0.2
79	*37,5	-
97	38,3	0.2

*Estimated value

Table 20. Fermentation result for a conventional process using *Bacillus* alpha-amylase (10+20 KNU/kg DS) and glucoamylase (500 AGU/kg DS)

Hour	L EtOH/100 kg grain	STDEV
0	-	-
25.5	22,5	1.3
48	33,9	0.7
69	*37,2	-
79	38,8	0.4
97	40,5	0.5

*Estimated value

Using a simulated industrial fermentation time in the interval of approximately 48-70 hours an equivalent or higher alcohol yield was obtained from the mash produced by the process of the invention than could be obtained from a mash produced by the more energy consuming two step hot slurry pre-liquefying and jet-cooking process.

Example 9

This example illustrates the conversion of granular wheat and common corn starch into glucose using a CGTase, a glucoamylase and an acid fungal alpha-amylase at 60°C.

Flasks with either 33% DS common corn or wheat granular starch were prepared and incubated as described in example 1. At zero hours the enzyme activities given in table 20 were dosed to the flasks. Samples were withdrawn after 24, 48, 72, and 96 hours and analyzed as described in example 1. The results are shown in table 21 and table 22.

Table 20. The enzyme activity levels used were:

CGTase	Glucoamylase	Acid fungal alpha-amylase
NU/g DS	AGU/g DS	AFAU/g DS
100.0	0.2	0.05

Table 21. Soluble dry solids as percentage of total dry substance using two different starch types.

Starch	24 hours	48 hours	72 hours	96 hours
Common corn	85.9	96.2	99.4	100.0
Wheat	95.7	98.9	99.6	100.0

Table 22. The DX of the soluble hydrolysate using the two different starch types.

Starch	24 hours	48 hours	72 hours	96 hours
Common corn	76.2	89.2	93.4	94.7
Wheat	86.2	92.4	93.6	94.4

CLAIMS

1. A one step process for producing a soluble starch hydrolysate, the process comprising subjecting a aqueous granular starch slurry at a temperature below the initial gelatinization temperature of said granular starch to the simultaneous action of;

   a first enzyme which;

   (a) is a member of the Glycoside Hydrolase Family13;

   (b) has alpha-1.4-glucosidic hydrolysis activity, and;

   (c) comprises a Carbohydrate-Binding Module Family 20,

   and at least one second enzyme which is a fungal alpha-amylase (EC 3.2.1.1), a beta-amylase (E.C. 3.2.1.2), or a glucoamylase (E.C.3.2.1.3).

2. The process of the preceding claim, wherein the starch slurry has 20-55% dry solids granular starch, preferably 25-40% dry solids granular starch, more preferably 30-35% dry solids, especially around 33% dry solids granular starch.

3. The process of any of the preceding claims, wherein at least 85%, 86% , 87%, 88%, 89% least 90%, 91%, 92%, 93% 94%, 95%, 96%, 97%, 98% or at least 99% of the dry solids of the granular starch is converted into a soluble starch hydrolysate.

4. The process of any of the preceding claims, wherein the first enzyme is of microbial origin, and preferably of bacterial origin.

5. The process of any of the preceding claims, wherein the first enzyme is a CGTase (EC 2.4.1.19).

6. The process of any of the preceding claims, wherein the first enzyme is a CGTase having a hydrolysis activity of at least 3.5, preferably at least 4, 4.5, 5, 6, 7, 8, 9,10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, or most preferably at least 23 micro mol per min/mg.

7. The process of any of the preceding claims, wherein the first enzyme is a CGTase having 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85% or even 90% homology to the amino acid sequence shown in figure 1 in Joergensen et al. (1997), Biotechnol. Lett. 19:1027-1031.

8. The process of any of the preceding claims, wherein the first enzyme is a maltogenic alpha-amylase (E.C. 3.2.1.133).

9. The process of any of the preceding claims, wherein the maltogenic alpha-amylase is derived from *Bacillus*, preferably from *B. stearothermophilus*.

10. The process of any of the preceding claims wherein the first enzyme is a maltogenic alpha-amylase having 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85% or even 90% homology to the amino acid sequence shown in SEQ ID NO:1 in WO9943794.

11. The process of any of the preceding claims, wherein the first enzyme is the maltogenic alpha-amylase having the amino acid sequence shown SEQ ID NO:1 in WO9943794 or a variant of said amino acid sequence disclosed in said patent.

12. The process of any of the preceding claims, wherein the first enzyme is a maltogenic alpha-amylase having a hydrolysis activity of at least 3.5, preferably at least 4, 4.5, 5, 6, 7, 8, 9, 10, 11, 12,13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 100, 200, 300, 400, 500, 600, or most preferably at least 700 micro mol per min/mg.

13. The process of any of the preceding claims, wherein the second enzyme is a fungal alpha-amylase, having 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85% or even 90% homology to the amino acid sequence shown in SEQ ID NO:10 in WO9623874.

14. The process of any of the preceding claims, wherein the second enzyme is a barley beta-amylase (E.C. 2.4.1.2), such as Spezyme® BBA 1500 or Spezyme® DBA from Genencor int.

15. The process of any of the preceding claims, wherein the second enzyme is a glucoamylase.

16. The process of any of the preceding claims, wherein the second enzyme is a glucoamylase derived from *Aspergillus oryzae*, such as a glucoamylase having 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85% or even 90% homology to the amino acid sequence shown in SEQ ID NO:2 in WO00/04136.

17. The process of any of the preceding claims wherein a third enzyme is present, said third enzyme being an alpha-amylase derived from a *Bacillus* sp., such as the enzymes, the variants and hybrids disclosed in WO99/19467, WO96/23874, WO97/41213, and WO99/19467.

18. The process of any of the preceding claims, wherein a third enzyme is present, said enzyme being an isoamylase or a pullulanase.

19. The process of any of the preceding claims, wherein the temperature is at least 58°C, 59°C, or more preferably at least 60°C.

20. The process of any of the preceding claims, wherein the pH is in the range of 3.0 to 7.0, preferably from 3.5 to 6.0, or more preferably from 4.0-5.0.

21. The process of any of the preceding claims, wherein the soluble starch hydrolysate has a DX of at least 94.5%, 95.0%, 95.5%, 96.0%, 96.5%, 97.0%, 97.5%, 98.0%, 98.5, 99.0% or at least 99.5%.

22. The process of any of the preceding claims, wherein the dominating saccharide in the soluble starch hydrolysate is glucose or maltose.

23. The process of any of the preceding claims, wherein the granular starch is obtained from tubers, roots, stems, or whole grain.

24. The process of any of the preceding claims, wherein the granular starch is obtained from cereals.

25. The process of any of the preceding claims, wherein the granular starch is obtained from corn, cobs, wheat, barley, rye, milo, sago, cassava, tapioca, sorghum, rice or potatoes.

26. The process of any of the preceding claims, wherein the granular starch is obtained from dry milling of whole grain or from wet milling of whole grain.

27. The process of any of the preceding claims, wherein the process is conducted in an ultrafiltration system and where the retentate is held under recirculation in presence of enzymes, raw starch and water and where the permeate is the soluble starch hydrolysate.

28. The process of any of the preceding claims, wherein the process is conducted in a continuous membrane reactor with ultrafiltration membranes and where the retentate is held under recirculation in presence of enzymes, raw starch and water and where the permeate is the soluble starch hydrolysate.

29. The process of any of the preceding claims, wherein the process is conducted in a continuous membrane reactor with microfiltration membranes and where the retentate is held under recirculation in presence of enzymes, raw starch and water and where the permeate is the soluble starch hydrolysate.

30. A process for production of high fructose starch-based syrup (HFSS), wherein a soluble starch hydrolysate of the process of any of the preceding claims is subjected to conversion into high fructose starch-based syrup (HFSS), such as high fructose corn syrup (HFCS).

31. A process for production of fuel or potable ethanol, wherein a soluble starch hydrolysate of the process of any of claims 1-29 is subjected to fermentation into ethanol.

32. The process of claim 31, wherein the fermentation step is carried out simultaneously or separately/sequential to the hydrolysis of the granular starch.

33. The process of any of the claims 31-32, wherein the process is conducted in an ultrafiltration system where the retentate is held under recirculation in presence of enzymes, raw starch, yeast, yeast nutrients and water and where the permeate is an ethanol containing liquid.

34. The process of any of the claims 31-33, wherein the process is conducted in a continuous membrane reactor with ultrafiltration membranes and where the retentate is held under recirculation in presence of enzymes, raw starch, yeast, yeast nutrients and water and where the permeate is an ethanol containing liquid.

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(74) Agent: MACKNIGHT, Kamrin, T.; Genencor International, Inc., 925 Page Mill Road, Palo Alto, CA 94304 (US).

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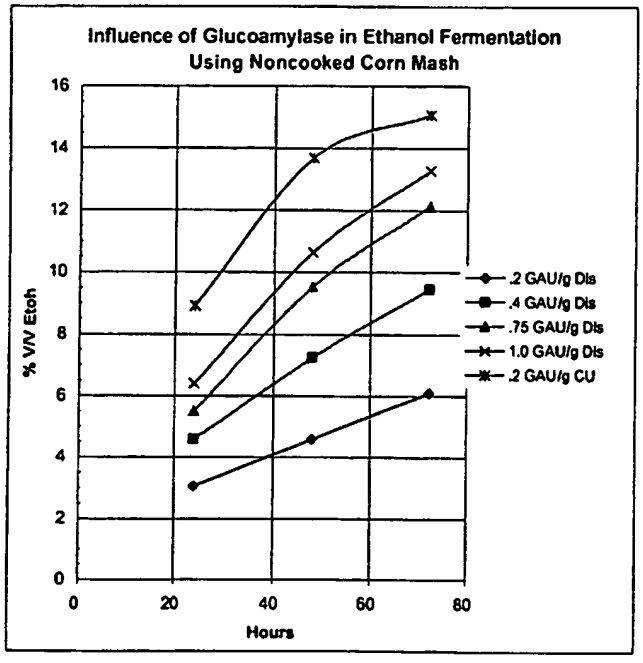
For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: METHODS FOR PRODUCING ETHANOL FROM CARBON SUBSTRATES



(57) Abstract: The present invention provides means for the production of desired end-products of in vitro and/or in vivo bioconversion of biomass-based feed stock substrates, including but not limited to such materials as starch and cellulose. In particularly preferred embodiments, the methods of the present invention do not require gelatinization and/or liquefaction of the substrate. In particularly preferred embodiments, the present invention provides means for the production of ethanol.

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METHODS FOR PRODUCING ETHANOL FROM CARBON SUBSTRATES

FIELD OF INVENTION

The present invention provides means for the production of desired end-products of *in vitro* and/or *in vivo* bioconversion of biomass-based feed stock substrates, including but not limited to such materials as starch and cellulose. In particularly preferred embodiments, the methods of the present invention do not require gelatinization and/or liquefaction of the substrate. In particularly preferred embodiments, the present invention provides means for the production of ethanol.

BACKGROUND OF THE INVENTION

Industrial fermentations predominantly utilize glucose as feed-stock for the production of proteins, enzymes and chemicals. These fermentations are usually batch, fed-batch, or continuous, and operate under conditions that are substrate-limited and/or designed to produce minimal by-products. As those in the art know, there are certain critical operating conditions that must be controlled during fermentation so as to optimize fermentation time, yield and efficiency.

Glucose is a natural, carbon based compound that is useful in a multitude of chemical and biological synthetic applications as a starting substrate. However, syrups that contain glucose purity levels of greater than 90% are relatively expensive. In addition, the presence of high glucose concentrations increases the susceptibility of the fermentation system to microbial contamination, thereby resulting in an adverse effect upon the production efficiency. Another disadvantage is that even the presence of low to moderate levels of glucose in the fermentation vat adversely affects the conversion of the glucose to the desired end product, for example by enzymatic inhibition and/or catabolite repression, and/or the growth of microorganisms. As a result, various attempts have been made to reduce

the costs of industrial fermentation, particularly in utilization of substrates that are less expensive than glucose. However, despite the development of numerous approaches, there remains a need in the art for economical, efficiently-utilized substrates for fermentation. Indeed, there is a great need in the art for methods that utilize a less expensive starting material than glucose to more efficiently produce a desired end-product.

SUMMARY OF THE INVENTION

The present invention provides means for the production of desired end-products of *in vitro* and/or *in vivo* bioconversion of biomass-based feed stock substrates, including but not limited to such materials as starch and cellulose. In particularly preferred embodiments, the methods of the present invention do not require gelatinization and/or liquefaction of the substrate. In particularly preferred embodiments, the present invention provides means for the production of ethanol. In some particularly preferred embodiments, the present invention provides means for the production of ethanol directly from granular starch, in which altered catabolite repression is involved.

In some embodiments, the present invention provides methods for producing ethanol in which the glucose concentration of the conversion medium is maintained at a low concentration, preferably below the threshold triggering catabolite repression and/or enzyme inhibition, so as to increase efficiency of the process by avoiding catabolic repressive and/or enzymatic inhibitive effects of glucose upon the enzymatic conversion of starch to ethanol.

In additional embodiments, the present invention provides methods for producing ethanol comprising the steps of contacting at least one carbon substrate with at least one substrate converting enzyme, to produce at least one intermediate, and then contacting at least one intermediate with at least one intermediate producing enzyme in a reactor vessel, wherein the at least one intermediate is substantially all bioconverted an end-product. In some preferred embodiments, a microorganism is used to achieve this bioconversion. By maintaining a low concentration of the intermediate in a conversion medium, the intermediate's catabolite repressive and/or enzymatic inhibitive effects are altered (*e.g.*, reduced).

The present invention also provides various levels of intermediate concentration, substrates, intermediates and steps of converting the intermediate to ethanol.

The present invention provides methods for producing an alcohol as an end-product comprising the steps of: contacting a carbon substrate and at least one substrate-converting enzyme to produce an intermediate; and contacting the intermediate with at least one intermediate-converting enzyme, wherein the intermediate is substantially all converted by the intermediate enzyme to alcohol. In some preferred embodiments, the intermediate-converting enzyme is a microbial enzyme. In alternative preferred embodiments, the intermediate-converting microbial enzyme is secreted by a microorganism that is in contact with the intermediate. In further embodiments, substrate-converting enzyme is a microbial enzyme. In some preferred embodiments, the substrate-converting microbial enzyme is secreted by a microorganism that is in contact with the substrate. In still other preferred embodiments, the intermediate-converting enzyme and the substrate-converting enzyme are produced by microorganisms of the same species. In alternative embodiments, the intermediate-converting enzyme and the substrate-converting enzyme are produced by microorganisms of the different species. In still further embodiments, the concentration level of the intermediate is maintained at a level below that which triggers catabolite repression effects upon the conversion of the intermediate to the end-product. In additional embodiments, concentration level of the intermediate is maintained at a level below that which triggers enzymatic inhibition effects upon the conversion of the intermediate to the end-product. In some preferred embodiments, the intermediate is converted to the end-product at a rate sufficient to maintain the concentration that is less than 0.25%. In yet other embodiments, the substrate is selected from the group consisting of biomass and starch. In some preferred embodiments, the biomass comprises corn solids. In some particularly preferred embodiments, intermediate is selected from the group consisting of hexoses and pentoses. In some embodiments, the hexose is glucose. In some embodiments, the substrate is cooked prior to its use in the present invention, while in other embodiments, the substrate is uncooked prior to its use in the present invention. In yet other embodiments, the step of contacting the substrate and substrate-converting enzyme further comprises bioconverting the

substrate to produce the intermediate. In most preferred embodiments, the alcohol end-product is ethanol. In still further embodiments, the step of contacting the substrate and at least one substrate-converting enzyme further comprises providing an amount of the substrate-converting enzyme at a concentration that produces the intermediate at a concentration that is less than or equal to the amount of the intermediate converted by at least one intermediate-converting enzyme. In some additional embodiments, at least one substrate-converting enzyme converts at least 50% of the substrate to the intermediate within 72 hours, while in other embodiments, at least one substrate-converting enzyme converts at least 90% of the substrate to the intermediate within 72 hours, and in some preferred embodiments, at least one substrate-converting enzyme converts at least 95% of the substrate to the intermediate within 72 hours. In still further embodiments, at least one substrate-converting enzyme and at least one intermediate-converting enzyme are obtained from a microorganism selected from the group consisting of *Rhizopus* and *Aspergillus*. In additional embodiments, the substrate-converting and/or intermediate-converting enzyme(s) are provided as a cell-free extract. In further preferred embodiments, the contacting steps take place in a reaction vessel, including but not limited to vats, bottles, flasks, bags, bioreactors, and any other receptacle suitable for conducting the methods of the present invention.

The present invention further provides methods for producing alcohol as an end-product comprising the steps of contacting a carbon substrate and at least one substrate-converting enzyme to produce an intermediate; and contacting the intermediate with at least one intermediate-converting enzyme, wherein the intermediate is substantially all converted by the intermediate enzyme to the alcohol end-product, and wherein the presence of the end-product does not inhibit the further production of the alcohol end-product. In some preferred embodiments, the intermediate-converting enzyme is a microbial enzyme. In alternative preferred embodiments, the intermediate-converting microbial enzyme is secreted by a microorganism that is in contact with the intermediate. In further preferred embodiments, the substrate-converting enzyme is a microbial enzyme. In still further embodiments, the substrate-converting microbial enzyme is secreted by a microorganism that is in contact with the substrate. In some embodiments,

intermediate-converting enzyme and the substrate-converting enzyme are produced by microorganisms of the same species, while in other embodiments, intermediate-converting enzyme and the substrate-converting enzyme are produced by microorganisms of the different species. In yet other embodiments, the substrate is selected from the group consisting of biomass and starch. In some preferred embodiments, the biomass comprises corn solids. In some particularly preferred embodiments, intermediate is selected from the group consisting of hexoses and pentoses. In some embodiments, the hexose is glucose. In some embodiments, the substrate is cooked prior to its use in the present invention, while in other embodiments, the substrate is uncooked prior to its use in the present invention. In some particularly preferred embodiments, the alcohol end-product is ethanol. In still further embodiments, the step of contacting the substrate and at least one substrate-converting enzyme further comprises providing an amount of the substrate-converting enzyme at a concentration that produces the intermediate at a concentration that is less than or equal to the amount of the intermediate converted by at least one intermediate-converting enzyme. In some additional embodiments, at least one substrate-converting enzyme converts at least 50% of the substrate to the intermediate within 72 hours, while in other embodiments, at least one substrate-converting enzyme converts at least 90% of the substrate to the intermediate within 72 hours, and in some preferred embodiments, at least one substrate-converting enzyme converts at least 95% of the substrate to the intermediate within 72 hours. In still further embodiments, at least one substrate-converting enzyme and at least one intermediate-converting enzyme are obtained from a microorganism selected from the group consisting of *Rhizopus* and *Aspergillus*. In additional embodiments, the substrate-converting and/or intermediate-converting enzyme(s) are provided as a cell-free extract. In further preferred embodiments, the contacting steps take place in a reaction vessel, including but not limited to vats, bottles, flasks, bags, bioreactors, and any other receptacle suitable for conducting the methods of the present invention.

The present invention further provides methods for producing an alcohol end-product comprising the steps of: contacting a carbon substrate and at least one substrate-converting enzyme to produce an intermediate; and contacting the

intermediate with at least one intermediate-converting enzyme, wherein the intermediate is substantially all converted by the intermediate enzyme to the alcohol end-product, and wherein the presence of the substrate does not inhibit the further production of the alcohol end-product. In some preferred embodiments, the intermediate-converting enzyme is a microbial enzyme. In alternative preferred embodiments, the intermediate-converting microbial enzyme is secreted by a microorganism that is in contact with the intermediate. In further preferred embodiments, the substrate-converting enzyme is a microbial enzyme. In still further embodiments, the substrate-converting microbial enzyme is secreted by a microorganism that is in contact with the substrate. In some embodiments, intermediate-converting enzyme and the substrate-converting enzyme are produced by microorganisms of the same species, while in other embodiments, intermediate-converting enzyme and the substrate-converting enzyme are produced by microorganisms of the different species. In yet other embodiments, the substrate is selected from the group consisting of biomass and starch. In some preferred embodiments, the biomass comprises corn solids. In some particularly preferred embodiments, intermediate is selected from the group consisting of hexoses and pentoses. In some embodiments, the hexose is glucose. In some embodiments, the substrate is cooked prior to its use in the present invention, while in other embodiments, the substrate is uncooked prior to its use in the present invention. In some particularly preferred embodiments, the alcohol end-product is ethanol. In still further embodiments, the step of contacting the substrate and at least one substrate-converting enzyme further comprises providing an amount of the substrate-converting enzyme at a concentration that produces the intermediate at a concentration that is less than or equal to the amount of the intermediate converted by at least one intermediate-converting enzyme. In some additional embodiments, at least one substrate-converting enzyme converts at least 50% of the substrate to the intermediate within 72 hours, while in other embodiments, at least one substrate-converting enzyme converts at least 90% of the substrate to the intermediate within 72 hours, and in some preferred embodiments, at least one substrate-converting enzyme converts at least 95% of the substrate to the intermediate within 72 hours. In still further embodiments, at least one substrate-

converting enzyme and at least one intermediate-converting enzyme are obtained from a microorganism selected from the group consisting of *Rhizopus* and *Aspergillus*. In additional embodiments, the substrate-converting and/or intermediate-converting enzyme(s) are provided as a cell-free extract. In further preferred embodiments, the contacting steps take place in a reaction vessel, including but not limited to vats, bottles, flasks, bags, bioreactors, and any other receptacle suitable for conducting the methods of the present invention.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 provides a graph showing the ethanol results for the experiments described in Example 1.

Figure 2, Panels A, B and C provide graphs showing the ethanol results from uncooked ground corn fermentation using M1 (Panel A), CU (Panel B), and M1 with DISTILLASE® (Panel C).

Figure 3, Panels A, B and C provide graphs showing the ethanol results obtained in the experiments described in Example 3.

Figure 4 shows the response of ethanol to the amount of stillage added in both types of mashes.

Figure 5 shows the glucose profile after 72 hour of fermentation as described in Example 4.

Figure 6 is a plot of the disaccharides after 72 hours of fermentation with respect to stillage added (*See*, Example 4).

Figure 7 shows the levels of the higher sugars (*i.e.*, oligosaccharides greater than disaccharides) (*See*, Example 4).

Figure 8 shows the lactic acid level after 72 hours of fermentation (*See*, Example 4).

Figure 9 summarizes the glycerol levels after 72 hours of fermentation (*See*, Example 4).

DESCRIPTION OF THE INVENTION

The present invention provides means for the production of desired end-products of *in vitro* and/or *in vivo* bioconversion of biomass-based feed stock

substrates, including but not limited to such materials as starch and cellulose. In particularly preferred embodiments, the methods of the present invention do not require gelatinization and/or liquefaction of the substrate. In particularly preferred embodiments, the present invention provides means for the production of ethanol. In some particularly preferred embodiments, the present invention provides means for the production of ethanol directly from granular starch, in which altered catabolite repression is involved.

In particular, the present invention provides means for making ethanol in a manner that is characterized by having altered levels of catabolite repression and enzymatic inhibition, thus increasing the process efficiency. The methods of the present invention comprise the steps of contacting a carbon substrate and a substrate converting enzyme to produce an intermediate; and contacting the intermediate with an intermediate producing enzyme in a reactor vessel, wherein the intermediate is substantially all bioconverted by an end-product producing microorganism. By maintaining a low concentration of the intermediate in a conversion medium, the catabolite repressive or enzymatic inhibitive effects of the intermediate on the process are altered.

The present invention also provides methods in which starches or biomass and hydrolyzing enzymes are used to convert starch or cellulose to glucose. In addition, the present invention provides methods in which these substrates are provided at such a rate that the conversion of starch to glucose matches the glucose feed rate required for the respective fermentative product formation. Thus, the present invention provides key glucose-limited fermentative conditions, as well as avoiding many of the metabolic regulations and inhibitions.

In some preferred embodiments, the present invention provides means for making desired end-products, in which a continuous supply of glucose is provided under controlled rate conditions, providing such benefits as reduced raw material cost, lower viscosity, improved oxygen transfer for metabolic efficiency, improved bioconversion efficiency, higher yields, altered levels of catabolite repression and enzymatic inhibition, and lowered overall manufacturing costs.

As indicated above, there is a great need in the art for methods in which less expensive starting materials than glucose are used to efficiently produce a desired

end-product. As described in greater detail herein, the present invention provides methods involving such substrates, including starch (*e.g.*, corn and wheat starch) and biomass.

Starch is a plant-based fermentation carbon source. Corn starch and wheat starch are carbon sources that are much cheaper than glucose carbon feedstock for fermentation. Conversion of liquefied starch to glucose is known in the art and is generally carried out using enzymes such alpha-amylase, pullulanase, and glucoamylase. A large number of processes have been described for converting liquefied starch to the monosaccharide, glucose. Glucose has value in itself, and also as a precursor for other saccharides such as fructose. In addition, glucose may also be fermented to ethanol or other fermentation products. However the ability of the enzymatic conversion of a first carbon source to the intermediate, especially glucose, may be impaired by the presence of the intermediate.

For example, the typical methods used in Japanese sake brewing and alcoholic production use starch without cooking. However, these techniques require some special operations such as acidification of mash (pH 3.5), which prevents contamination of harmful microorganisms. Furthermore, these methods require a longer period of the time for the saccharification and fermentation than the present invention. In addition, these methods require complex process steps such as dialysis of a fermented broth and are too cumbersome to utilize in the general production of products via fermentation.

The use of soluble dextrins and glucose as feed-stock in fermentations have various drawbacks, including high processing cost, problems associated with viscosity and oxygen transfer. In addition, in comparison to the present invention, these methods produce lower yields of the desired products and more problems associated with the formation of by-products. Indeed, the costs of converting starch or biomass to dextrins are substantial and involve high energy input, separate reactor tanks, more time, a detailed bioprocess operation, incomplete saccharification, back-reaction, and enzymes associated with the typical pre-fermentation saccharification step. These problems have led to a number of attempts to provide methods for conversion directly to starch within one reaction vessel or container and at lower temperatures. Biotransformation of a

carbohydrate source to 1,3-propanediol in mixed cultures is described in U.S. Patent No. 5,599,689 (Haynie, *et al.*). The method described by Haynie *et al.*, involves mixing a glycerol (*i.e.*, an intermediate) producing organism with a diol producing organism (*i.e.*, an end-product), contacting the mixed culture medium with a carbon substrate and incubating the mixed culture medium to produce the desired end-product, 1,3-propanediol. In U.S. Patent No. 4,514,496, Yoshizuma describes methods that involve maintaining the concentration of the material in the slurry relative the mashing liquid to produce alcohol by fermentation without cooking (*i.e.*, without high temperature liquefaction before saccharization. Nonetheless, these methods lack the efficiency and economical advantages provided by the present invention.

The present invention provides methods for producing end-products, including organic acids (*e.g.*, gluconic acid, ascorbic acid intermediates, succinic acid, citric acid, acetic acid, gluconic acid, and lactic acid), amino acids, antibiotics, enzymes and organic solvents (*e.g.*, 1,3-propanediol, butanediol, and acetone), glycerol, ethanol are provided. In some preferred embodiments, the methods comprise the steps of contacting at least one carbon substrate with at least one substrate converting enzyme to produce at least one intermediate; and contacting at least one intermediate with an intermediate producing enzyme in a reactor vessel, wherein at least one intermediate is substantially completely bioconverted an end-product. In some preferred embodiments, this bioconversion is achieved by microorganisms. By maintaining a low concentration of the intermediate in a conversion medium, the intermediate's catabolite repressive and/or enzymatic inhibitive effects are altered (*e.g.*, reduced). The present invention also provides various levels of intermediate concentration, substrates, intermediates and steps of converting the intermediate to the desired end-product (*e.g.*, ethanol).

Definitions

Unless defined otherwise herein, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Various references (*See e.g.*, Singleton, *et al.*, DICTIONARY OF MICROBIOLOGY AND MOLECULAR BIOLOGY, 2D ED., John Wiley and

Sons, New York [1994]; and Hale and Marham, THE HARPER COLLINS DICTIONARY OF BIOLOGY, Harper Perennial, NY [1991]) provide general definitions of many of the terms used herein. Furthermore, all patents and publications, including all sequences disclosed within such patents and publications, referred to herein are expressly incorporated by reference.

Although any methods and materials similar or equivalent to those described herein find use in the practice of the present invention, preferred methods and materials are described herein. Numeric ranges are inclusive of the numbers defining the range. Unless otherwise indicated, nucleic acids are written left to right in 5' to 3' orientation; amino acid sequences are written left to right in amino to carboxy orientation, respectively. It is to be understood that this invention is not limited to the particular methodology, protocols, and reagents described, as these may vary.

The headings provided herein are not limitations of the various aspects or embodiments of the invention that can be had by reference to the specification as a whole. Furthermore, the terms defined immediately below are more fully defined by reference to the Specification as a whole.

As used herein, the term "carbon substrate" refers to a material containing at least one carbon atom which can be enzymatically converted into an intermediate for subsequent conversion into the desired carbon end-product. Exemplary carbon substrates include, but are not limited to biomass, starches, dextrins and sugars.

As used herein, "biomass" refers to cellulose- and/or starch-containing raw materials, including but not limited to wood chips, corn stover, rice, grasses, forages, perrie-grass, potatoes, tubers, roots, whole ground corn, cobs, grains, wheat, barley, rye, milo, brans, cereals, sugar-containing raw materials (*e.g.*, molasses, fruit materials, sugar cane or sugar beets), wood, and plant residues. Indeed, it is not intended that the present invention be limited to any particular material used as biomass. In preferred embodiments of the present invention, the raw materials are starch-containing raw materials (*e.g.*, cobs, whole ground corns, corns, grains, milo, and/or cereals, and mixtures thereof). In particularly preferred embodiments, the term refers to any starch-containing material originally obtained from any plant source.

As used herein, "starch" refers to any starch-containing materials. In particular, the term refers to various plant-based materials, including but not limited to wheat, barley, potato, sweet potato, tapioca, corn, maize, cassava, milo, rye, and brans. Indeed, it is not intended that the present invention be limited to any particular type and/or source of starch. In general, the term refers to any material comprised of the complex polysaccharide carbohydrates of plants, comprised of amylose and amylopectin, with the formula $(C_6H_{10}O_5)_x$, wherein "*x*" can be any number.

As used herein, "cellulose" refers to any cellulose-containing materials. In particular, the term refers to the polymer of glucose (or "cellobiose"), with the formula $(C_6H_{10}O_5)_x$, wherein "*x*" can be any number. Cellulose is the chief constituent of plant cell walls and Is among the most abundant organic substances in nature. While there is a β-glucoside linkage in cellulose, there is a an α-glucoside linkage in starch. In combination with lignin, cellulose forms "lignocellulose."

As used herein, the term "corn solids" refers to ground materials from corn, including but not limited to kernels, bran and cobs.

As used herein, "intermediate" refers to a compound that contains at least one carbon atom into which the carbon substrates are enzymatically converted. Exemplary intermediates include, but are not limited to pentoses (*e.g.*, xylose, arabinose, lyxose, ribose, ribulose, xylulose); hexoses (*e.g.*, glucose, allose, altrose, mannose, gulose, idose, galactose, talose, psicose, fructose, sorbose, and tagatose); and organic acids thereof.

As used herein, the term "enzymatic conversion" refers to the modification of a carbon substrate to an intermediate or the modification of an intermediate to an end-product by contacting the substrate or intermediate with an enzyme. In some embodiments, contact is made by directly exposing the substrate or intermediate to the appropriate enzyme. In other embodiments, contacting comprises exposing the substrate or intermediate to an organism that expresses and/or excretes the enzyme, and/or metabolizes the desired substrate and/or intermediate to the desired intermediate and/or end-product, respectively.

As used herein, the term "starch hydrolyzing enzyme " refers to any enzyme that is capable of converting starch to the intermediate sugar (*e.g.*, a hexose or pentose).

As used herein, "monosaccharide" refers to any compound having an empirical formula of $(CH_2O)_n$, wherein n is 3-7, and preferably 5-7. In some embodiments, the term refers to "simple sugars" that consist of a single polyhydroxy aldehyde or ketone unit. The term encompasses, but is not limited to such compounds as glucose, galactose, and fructose.

As used herein, "disaccharide" refers to any compound that comprises two covalently linked monosaccharide units. The term encompasses, but is not limited to such compounds as sucrose, lactose and maltose.

As used herein, "oligosaccharide" refers to any compound having 2 - 10 monosaccharide units joined in glycosidic linkages. In some preferred embodiments, the term refers to short chains of monosaccharide units joined together by covalent bonds.

As used herein, "polysaccharide" refers to any compound having multiple monosaccharide units joined in a linear or branched chain. In some preferred embodiments, the term refers to long chains with hundreds or thousands of monosaccharide units. Some polysaccharides, such as cellulose have linear chains, while others (*e.g.*, glycogen) have branched chains. Among the most abundant polysaccharides are starch and cellulose, which consist of recurring glucose units (although these compounds differ in how the glucose units are linked).

As used herein, "culturing" refers to fermentative bioconversion of a carbon substrate to the desired end-product within a reactor vessel. In particularly preferred embodiments, culturing involves the growth of microorganisms under suitable conditions for the production of the desired end-product(s).

As used herein, the term "saccharification" refers to converting a directly unusable polysaccharide to a useful sugar feed-stock for bioconversion or fermentative bioconversion.

As used herein, the term "fermentation" refers to the enzymatic and anaerobic breakdown of organic substances by microorganisms to produce simpler organic products. In preferred embodiments, fermentation refers to the utilization of

carbohydrates by microorganisms (*e.g.*, bacteria) involving an oxidation-reduction metabolic process that takes place under anaerobic conditions and in which an organic substrate serves as the final hydrogen acceptor (*i.e.*, rather than oxygen). Although fermentation occurs under anaerobic conditions, it is not intended that the term be solely limited to strict anaerobic conditions, as fermentation also occurs in the presence of oxygen.

As used herein, the terms "substantially all consumed" and "substantially all bioconverted" refer to the maintenance of a low level of intermediate in a conversion medium which adversely affects the enzymatic inhibition, oxygen transfer, yield, byproduct minimization or catabolite repression effects of the intermediate (*e.g.*, a hexose), upon the ability of the intermediate converting enzyme to convert the intermediate to the end-product or another intermediate and/or the ability of the substrate converting enzyme to convert the substrate to the intermediate.

As used herein, the terms "bioconversion" and "bioconverted" refer to contacting a microorganism with the carbon substrate or intermediate, under conditions such that the carbon substrate or intermediate is converted to the intermediate or desired end-product, respectively. In some embodiments, these terms are used to describe the production of another intervening intermediate in *in vitro* methods in which biocatalysts alone are used. In some preferred embodiments, the terms encompass metabolism by microorganisms and/or expression or secretion of enzyme(s) that achieve the desired conversion.

As used herein, the terms "conversion media" and "conversion medium" refer to the medium/media in which the enzymes and the carbon substrate, intermediate and end-products are in contact with one another. These terms include, but are not limited to fermentation media, organic and/or aqueous media dissolving or otherwise suspending the enzymes and the carbon substrate, intermediate and end-products. In some embodiments, the media are complex, while in other preferred embodiments, the media are defined.

As used herein, the term "end-product" refers to any carbon-source derived molecule product which is enzymatically converted from the intermediate. In particularly preferred embodiments, the methods of the present invention are used

in order to produce a "desired end-product" (*i.e.*, the product that is intended to be produced through the use of these methods). In particularly preferred embodiments, the term refers to an alcohol, particularly ethanol.

As used herein, "low concentration" refers to a concentration level of a compound that is less than that would result in the production of detrimental effects due to the presence of the compound. In particularly preferred embodiments, the term is used in reference to the concentration of a particular intermediate below which the detrimental effects of catabolite suppression and/or enzyme inhibition are observed. In some embodiments, the term refers to the concentration level of a particular intermediate above which triggers catabolite repression and/or enzymes inhibition by substrate and/or products.

As used herein, the phrase "maintained at a level below which triggers catabolite repression effects" refers to maintaining the concentration of an intermediate to below that level which triggers catabolite repression.

As used herein, the term "reduces catabolite repression" means conditions under which the effects of catabolite repression are produced. In preferred embodiments, the term refers to conditions in which the intermediate concentration is less than that threshold which triggers catabolite repressive effects.

As used herein, the term "reduces enzyme inhibition" means conditions under which the inhibition of an enzyme is reduced as compared to the inhibition of the enzyme under usual, standard conditions. In preferred embodiments of the present invention, the term refers to conditions in which the concentration of an intermediate, substrate and/or product of the enzyme reaction is less than that threshold which triggers enzyme inhibition.

As used herein, the term "substrate converting enzyme" refers to any enzyme that converts the substrate (*e.g.*, granular starch) to an intermediate, (*e.g.*, glucose). Substrate converting enzymes include, but are not limited to alpha-amylases, glucoamylases, pullulanases, starch hydrolyzing enzymes, and various combinations thereof.

As used herein, the term "intermediate converting enzyme" refers to any enzyme that converts an intermediate (*e.g.*, D-glucose, D-fructose, etc.), to the desired end-product. In preferred embodiments, this conversion is accomplished

through hydrolysis, while in other embodiments, the conversion involves the metabolism of the intermediate to the end-product by a microorganism. However, it is not intended that the present invention be limited to any particular enzyme or means of conversion. Indeed, it is intended that any appropriate enzyme will find use in the various embodiments of the present invention.

As used herein, "yield" refers to the amount of end-product or intermediate produced using the methods of the present invention. In some preferred embodiments, the yield produced using the methods of the present invention is greater than that produced using methods known in the art. In some embodiments, the yield refers to the volume of the end-product or intermediate, while in other embodiments, the term is used in reference to the concentration of the end-product or intermediate in a composition.

As used herein, "byproduct formation" refers to the production of products that are not desired. In some preferred embodiments, the present invention provides methods that avoid or reduce the production of byproducts, as compared to methods known in the art.

As used herein, the term "enzymatic inhibition" refers to loss of enzyme activity by either physical or biochemical effects on the enzyme. In some embodiments, inhibition results from the effects of the product formed by the enzyme activity, while in other embodiments, inhibition results from the action of the substrate or intermediate on the enzyme.

As used herein, "enzyme activity" refers to the action of an enzyme on its substrate. In some embodiments, the enzyme activity is quantitated using means to determine the conversion of the substrate to the intermediate, while in other embodiments, the conversion of the substrate to the end-product is determined, while in still further embodiments, the conversion of the intermediate to the end-product is determined.

As used herein, the term "enzyme unit" refers to the amount of enzyme which converts 1 micromole of substrate per minute to the substrate product at optimum assay conditions (unless otherwise noted). In some embodiments, commercially available enzymes (*e.g.*, SPEZYME®, DISTALLASE®, OPTIMAX®; Genencor International) find use in the methods of the present invention.

As used herein, the term "glucoamylase unit" (GAU) is defined as the amount of enzyme required to produce one micromole of glucose per minute under assay conditions of 40° C. and pH 5.0 or under the alternative assay conditions of 25° and pH 7.0.

As used herein, the term "glucose oxidase unit" (GOU) is defined as the amount of enzyme required to oxidize one micromole of D-glucose per minute under assay conditions of 25° C. and pH 7.0, to gluconic acid.

As used herein, the term "catalase units" (CU) is defined as the amount of enzyme required to decompose 1 micromole of hydrogen peroxide per minute under assay conditions of 25° C. and pH 7.0.

As used herein, one AG unit (AGU) is the amount of enzyme which splits one micromole of maltose per minute at 25° C. and pH 4.3. In some embodiments of the present invention, a commercially available liquid form of glucoamylase (OPTIDEX® L-400; Genencor International) with an activity of 400 GAU per ml is used. In alternative embodiments, a commercially available liquid form of glucoamylase (AMG NOVO 150) has an activity of 150 AGU per ml finds use.

As used herein, the terms "starch hydrolyzing unit" and "raw starch hydrolyzing unit" (RHU) are defined as being the amount of enzyme required to produce one gram of glucose per minute from starch, under the assay conditions of 25° C. and pH 5.0.

As used herein, "carbon end-product" means any carbon product produced from the carbon intermediate, wherein the substrate contains at least one carbon atom (*i.e.*, a carbon substrate).

As used herein, "carbon intermediate" refers to the carbon-containing compounds that are produced during the conversion of a carbon-containing substrate to a carbon end-product.

As used herein, " enzymatically controlled" means regulating the amount of carbon intermediate produced from the carbon substrate by altering the amount or activity of the enzyme used in the reaction.

As used herein, "microorganism" refers to any organism with cells that are typically considered to be microscopic, including such organisms as bacteria, fungi (yeasts and molds), rickettsia, and protozoa. It is not intended that the present

invention be limited to any particular microorganism(s) or species of microorganism(s), as various microorganisms and microbial enzymes are suitable for use in the present invention. It is also not intended that the present invention be limited to wild-type microorganisms, as microorganisms and microbial enzymes produced using recombinant DNA technologies also find use in the present invention.

As used herein, "microbial enzyme" refers to any enzyme that is produced by a microorganism. As used herein, a "microbial intermediate-converting enzyme" is an enzyme that converts an intermediate to an end-product, while a "microbial substrate-converting enzyme" is an enzyme that converts a substrate to an intermediate or directly converts a substrate to an end-product (*i.e.*, there is not intermediate compound).

As used herein, the term "ethanologenic microorganism" refers to a microorganism with the ability to convert a sugar or oligosaccharide to ethanol. Ethanologenic microorganisms are known in the art and include ethanologenic bacteria. The microorganisms are ethanologenic by virtue of their ability to express one or more enzymes that individually or together, convert a sugar to ethanol.

As used herein, the terms "ethanol producer" and "ethanol producing organism" refer to any organism or cell that is capable of producing ethanol from a hexose or a pentose. Generally, ethanol producing cells contain an alcohol dehydrogenase and pyruvate decarboxylase.

As used herein, "antimicrobial" refers to any compound that kills or inhibits the growth of microorganisms.

As used herein, the term "linked culture" refers to a fermentation system that employs at least two cell cultures, in which the cultures are added sequentially. In most embodiments of linked systems, a primary culture or a set of primary cultures is grown under optimal fermentation conditions for the production of a desired intermediate (*i.e.*, the intermediate is released into the culture media to produce a "conditioned medium"). Following the fermentation of the primary culture, the conditioned medium is then exposed to the secondary culture(s). The secondary cultures then convert the intermediate in the conditioned media to the desired end-product. In some embodiments of the present invention, the primary cultures are

typically glycerol producers and the secondary cultures are 1,3-propanediol producers.

As used herein, "mixed culture" refers to the presence of any combination of microbial species in a culture. In some preferred embodiments, the mixed culture is grown in a reaction vessel under conditions such that the interaction of the individual metabolic processes of the combined organisms results in a product which neither individual organism is capable of producing. It is not intended that the present invention be limited to mixed cultures comprising a particular number of microbial species.

As used herein, "conditioned media" refers to any fermentation media suitable for the growth of microorganisms that has been supplemented by organic byproducts of microbial growth. In preferred embodiments of the present invention, conditioned media are produced during fermentation of linked cultures wherein glycerol producing cells secrete glycerol into the fermentation media for subsequent conversion to 1,3-propanediol.

As used herein, "oxygen uptake rate" ("OUR") refers to the determination of the specific consumption of oxygen within the reactor vessel. Oxygen consumption can be determined using various on-line measurements known in the art. In one embodiment, the OUR (mmol/(liter*hour)) is determined by the following formula: ((Airflow (standing liters per minute) / Fermentation weight (weight of the fermentation broth in kilograms)) X supply O_2 X broth density X (a constant to correct for airflow calibration at 21.1 C instead of standard 20.0 C)) minus ([airflow /fermentation weight] x [offgas O_2/offgas N_2] X supply N_2 X broth density X constant).

As used herein, "carbon evolution rate" ("CER") refers to the determination of how much CO_2 is produced within the reactor vessel during fermentation. Usually, since no CO_2 is initially or subsequently provided to the reaction vessel, any CO_2 is assumed to be produced by the fermentation process occurring within the reaction vessel. "Off-gas CO_2" refers to the amount of CO_2 measured within the reactor vessel, usually by mass spectroscopic methods known in the art.

As used herein, the term "enhanced" refers to improved production of proteins of interest. In preferred embodiments, the present invention provides

enhanced (*i.e.*, improved) production and secretion of a protein of interest. In these embodiments, the "enhanced" production is improved as compared to the normal levels of production by the host (*e.g.*, wild-type cells). Thus, for heterologous proteins, basically any expression is enhanced, as the cells normally do not produce the protein.

As used herein, the terms "isolated" and "purified" refer to a nucleic acid or amino acid that is removed from at least one component with which it is naturally associated.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides means for the production of desired end-products of *in vitro* and/or *in vivo* bioconversion of biomass-based feed stock substrates, including but not limited to such materials as starch and cellulose. In particularly preferred embodiments, the methods of the present invention do not require gelatinization and/or liquefaction of the substrate. In particularly preferred embodiments, the present invention provides means for the production of ethanol. In some particularly preferred embodiments, the present invention provides means for the production of ethanol directly from granular starch, in which altered catabolite repression is involved.

In preferred embodiments, the present invention provides dramatic improvements in the process for directly converting a commonly available carbon substrate (*e.g.*, biomass and/or starch) into an intermediate, preferably, an intermediate that is readily convertible into a multitude of desired end-products, including alcohols such as ethanol. In particularly preferred embodiments, the present invention provides means for dramatically improving the processes for directly converting granular starch into glucose, an intermediate readily convertible into a ethanol.

In alternative embodiments, the present invention provides means for dramatic improvements in the process for converting starch or cellulose into glucose, which in turn is converted into the desired end-product. By maintaining the presence of the intermediate at a low concentration within the conversion media, overall efficiency of the production is improved. In some embodiments, enzymatic

inhibition and/or catabolite repression, oxygen uptake demand, and/or byproduct formation are reduced. In additional preferred embodiments, the present invention provides means for dramatic improvements in the non-cooking conversion of granular starch into glucose, which in turn is converted into the desired end-product.

In some preferred embodiments, the maintenance of minimal intermediate concentrations is achieved by maintaining the concentration of the intermediate at a low concentration. In one embodiment, the concentration of the intermediate is less than or equal to 0.25% by weight volume of the medium (*e.g.*, 0.25% to 0.00001% by weight volume). In other embodiments, the concentration of the intermediate is less than or equal to 0.20%, 0.10%, 0.05%, or 0.01% by weight volume (*e.g.*, 0.20% to 0.00001%, 0.10% to 0.00001% 0.05% to 0.00001%, 0.01% to 0.00001%, respectively). Alternatively, the intermediate concentration is maintained at less than or equal to a concentration of 2.0 µmolar in the conversion media. In another embodiment, the concentration is maintained at less than or equal to 1.0 µmolar. In still another embodiment, the concentration of the intermediate is maintained at a concentration of less than or equal to 0.75 µmolar. In any event, maintaining a low concentration means maintaining the concentration of the intermediate below the threshold that results in enzyme inhibition (*i.e.*, enzyme inhibitive effects), catabolite repression (*i.e.*, catabolite repressive effects).

In further embodiments, the maintenance of a minimal concentration is achieved by maintaining the rate of conversion of the substrate to the intermediate at less than or equal to the rate of conversion of the intermediate to the end-product. While it is recognized that the conversion of the substrate to the intermediate is necessarily rate limiting for the conversion of the intermediate to the end-product, by providing sufficient intermediate converting enzymes for the conversion of substantially all of the intermediate produced by the first enzymatic conversion from the carbon substrate, substantially all of the intermediate is converted to the end-product as fast as it is converted from the starting substrate to minimize the presence of the intermediate in the conversion medium. Exemplary methods of providing such excessive intermediate conversion include providing an excess of intermediate converting enzyme, increasing the enzyme activity of the intermediate converting enzyme, and/or decreasing the activity of the substrate

converting enzyme to convert the intermediate to end-product as quickly as it is converted from the substrate. As the actual rate of conversion is contemplated to vary with the specific end product produced, some variation in the amount and experimentation in determining the amount are contemplated. However guidelines for making these determinations are provided herein.

In some embodiments of the present invention, the conversion or consumption rate of the intermediate was determined by the calculating the amount of organism present in the mixed media, taking into consideration the other physical parameters of the mixed media, and multiplying that amount by the generally known conversion rate. This provides a rate of conversion of the intermediate, (*e.g.*, glucose), to the end-product. In some embodiments, this conversion of the intermediate to the desired end product is by conversion or bioconversion of the intermediate to the end-product by a naturally occurring organism or one mutated to provide such bioconversion. Another embodiment of the conversion from intermediate to end product involves the use of an enzymatic conversion by a known enzyme to the desired end-product using known enzymatic conversion methods. For example, in some embodiments, the conversion of glucose to a desired end product (*e.g.*, propanediol, succinic acid, gluconic acid, lactic acid, amino acid, antimicrobials, ethanol, ascorbic acid intermediates and /or ascorbic acid) is accomplished by the addition of an amount of an enzyme known to convert glucose to the specified end product desired.

Once the conversion rate of the intermediate to the desired end product is determined, the limit of the conversion of the carbon substrate to the intermediate can be determined in the same manner. By calculating the upper limit of the intermediate to end product conversion, the conversion rate of the carbon substrate to intermediate can be determined, the main consideration being that the intermediate concentration levels in the conversion media are maintained at a sufficiently low level to adversely effect the normally catabolite repressive/enzymatic inhibitory effects of the intermediate. In one embodiment, this is accomplished by maintaining the conversion rate of the intermediate to the end product in excess or equal to the rate of conversion of the carbon substrate to the intermediate. Thus, the present invention provides means for increasing the conversion rate to the end

product, as well as means for restricting the conversion of the carbon substrate to the intermediate.

Another method for determining whether the rate of conversion of the intermediate to the end product is greater than or equal to the production of the intermediate from the carbon substrate is to measure the weight percentage of the intermediate in the reactor vessel. The amount of the intermediate present in the reactor vessel can be determined by various known methods, including, but not limited to direct or indirect measurement of the amount of intermediate present in the reactor vessel. Direct measurement can be by periodic assays of the reactor vessel, using assays known to identify the amount of intermediate and or end-product in the vessel. In addition, direct measurement of the amounts of intermediates within the reactor vessel include on-line gas, liquid and/or high performance liquid chromatography methodologies known in the art

Indirect measurement of the levels of intermediate or end-products produced can be assessed by the measurement of oxygen uptake or carbon dioxide production, using methods known in the art (*e.g.*, by determining the oxygen uptake rate and/or the carbon evolution rate).

Substrates

The substrates of the present invention are carbon-based compounds that can be converted enzymatically to intermediate compounds. Suitable substrates include, but are not limited to processed materials that contain constituents which can be converted into sugars (*e.g.*, cellulosic biomass, glycogen, starch and various forms thereof, such as corn starch, wheat starch , corn solids and wheat solids). During the development of the present invention good results were obtained with corn starch and wheat starch, although other sources, including starches from grains and tubers (*e.g.*, sweet potato , potato , rice and cassava starch) also find use with the present invention. Various starches are commercially available. For example, corn starches are available from Cerestar, Sigma, and Katayama Chemical Industry Co. (Japan); wheat starches are available from Sigma; sweet potato starch is available from Wako Pure Chemical Industry Co. (Japan); and

potato starch is available from Nakari Chemical Pharmaceutical Co. (Japan). A particularly useful carbon substrate is corn starch. In some embodiments of the present invention, granular starch is used in a slurry having a percentage of starch between about 20% and about 35%. Preferably, the starch is in a concentration between about 10% and about 35%. In some particularly preferred embodiments, the range for percent starch is between 30% and 32%. In addition to granular starch, other carbon substrate sources find use in the present invention include, but are not limited to biomass, polysaccharides, and other carbon based materials capable of being converted enzymatically to an intermediate.

Fermentable sugars can be obtained from a wide variety of sources, including lignocellulosic material. Lignocellulose material can be obtained from lignocellulosic waste products (*e.g.*, plant residues and waste paper). Examples of suitable plant residues include but are not limited to any plant material such as stems, leaves, hulls, husks, cobs and the like, as well as corn stover, begasses, wood, wood chips, wood pulp, and sawdust. Examples of paper waste include but are not limited to discarded paper of any type (*e.g.*, photocopy paper, computer printer paper, notebook paper, notepad paper, typewriter paper, and the like), as well as newspapers, magazines, cardboard, and paper-based packaging materials. The conditions for converting sugars to ethanol are known in the art. Generally, the temperature is between about 25 ° C. and 35 ° C (*e.g.*, between 25° and 35°, and more particularly at 30° C). Useful pH ranges for the conversion medium are provided between about 4.0 and 6.0, between 4.5 and 6.0, and between pH 5.5 and 5.8. However, it is not intended that the present invention be limited to any particular temperature and/or pH conditions as these conditions are dependent upon the substrate(s), enzyme(s), intermediate(s), and/or end-product(s) involved.

Enzymes

In some preferred embodiments of the present invention, enzymes that are substrate-converting enzymes (*i.e.*, enzymes that are able to first convert the carbon substrate into the carbon intermediate), and intermediate converting enzymes (*i.e.*, enzymes that are able to convert the resulting intermediate into an intervening intermediate and/or the desired end-product) both find use in the

present invention. Enzymes that find use in some embodiments of the present invention to convert a carbon substrate to an intermediate include, but are not limited to alpha-amylase, glucoamylase, starch hydrolyzing glucoamylase, and pullulanase. Enzymes that find use in the conversion of an intermediate to an end-product depend largely on the actual desired end-product. For example enzymes useful for the conversion of a sugar to 1,3-propanediol include, but are not limited to enzymes produced by *E. coli* and other microorganisms. For example enzymes useful for the conversion of a sugar to lactic acid include, but are not limited to those produced by *Lactobacillus* and *Zymomonas*. Enzymes useful for the conversion of a sugar to ethanol include, but are not limited to alcohol dehydrogenase and pyruvate decarboxylase. Enzymes useful for the conversion of a sugar to ascorbic acid intermediates include, but are not limited to glucose dehydrogenase, gluconic acid dehydrogenase, 2,5-diketo-D-gluconate reductase, and various other enzymes. Enzymes useful for the conversion of a sugar to gluconic acid include, but are not limited to glucose oxidase and catalase.

In some preferred embodiments, the alpha-amylase used in some methods of the present invention is generally an enzyme which effects random cleavage of alpha-(1-4) glucosidic linkages in starch. In most embodiments, the alpha-amylase is chosen from among the microbial enzymes having an E. C. number E. C. 3.2.1.1 and in particular E. C. 3.2.1.1-3. In some preferred embodiments, the alpha-amylase is a thermostable bacterial alpha-amylase. In most particularly preferred embodiments, the alpha-amylase is obtained or derived from *Bacillus* species. Indeed, during the development of the present invention good results were obtained using the SPEZYME® alpha-amylase obtained from *Bacillus licheniformis* (Genencor). In other embodiments, black-koji amylase described in alcoholic fermentation from starch such as corn and cassava without precooking (Ueda *et al.*, J. Ferment. Technol., 50:237-242 [1980]; and Ueda *et al*, J. Ferment. Technol., 58:237-242 [1980]) find use in the present invention.

As understood by those in the art, the quantity of alpha-amylase used in the methods of the present invention will depend on the enzymatic activity of the alpha-amylase and the rate of conversion of the generated glucose by the end-product converter. Generally an amount between 0.001 and 2.0 ml of a solution of the

alpha-amylase is added to 1000 gm of raw materials, although in some embodiments, it is added in an amount between 0.005 and 1.5 ml of such a solution. In some preferred embodiments, it is added in an amount between 0.1 and 1.0 ml of such a solution. In further embodiments, other quantities are utilized. For example, generally an amount between 0.01 and 1.0 kg of SPEZYME® FRED (Genencor) is added to one metric ton of starch. In some embodiments, the enzyme is added in an amount between 0.4 to 0.6 kg, while in other embodiments, it is added in an amount between 0.5 and 0.6 kg of SPEZYME® FRED/metric ton of starch..

In preferred embodiments of the present invention, the glucoamylase is an enzyme which removes successive glucose units from the non-reducing ends of starch. The enzyme can hydrolyze both the linear and branched glucosidic linkages of starch, amylose and amylopectin. In most embodiments, the glucoamylase used in the methods of the present invention are microbial enzymes. In some preferred embodiments, the glucoamylase is a thermostable fungal glucoamylase, such as the *Aspergillus* glucoamylase. Indeed, during the development of the present invention, good results were obtained using the DISTALLASE® glucoamylase derived from *Aspergillus niger* (Genencor). Glucoamylase preparations from *Aspergillus niger* have also been used without the use of precooking (*See*, Ueda *et al*, Biotechnol. Bioeng., 23:291[1981]). Three glucoamylases have been selectively separated from *Aspergillus awamori var. kawachi* for use in hydrolyzing starch (*See*, Hayashida, Agr. Biol. Chem., 39:2093-2099 [1973]). Alcoholic fermentation of sweet potato by *Endomycopsis fibuligoeu* glucoamylase without cooking has also been described (Saha *et al.,* Biotechnol. Bioeng., 25:1181-1186 [1983]). Another enzyme that finds use in the present invention is glucoamylase (EC 3.2.1.3), an enzyme that hydrolyzes the alpha.-1,4-glucoside chain progressively from the non-reducing terminal end. This enzyme also hydrolyzes the alpha-1,6-glucoside chain. Glucoamylase is secreted from fungi of the genera *Aspergillus*, *Rhizopus* and *Mucor* also find use in the methods of the present invention. These enzymes further find use in glucose production and quantitative determination of glycogen and starch. Glucoamylase preparations obtained from *E. fibuligera* (IFO 0111) have been used to contact sweet potato starch for alcoholic fermentation (*See*,

Saha *et al.*, Biotechnol. Bioeng., 25:1181-1186 [1983]). One of this enzyme's major applications is as a saccharifying agent in the production of ethyl alcohol from starchy materials. However, as with the other glucoamylases described herein, this enzyme also finds use in the methods of the present invention.

Additional glucoamylases that find use in the methods of the present invention include those obtained from the genera *Rhizopus and Humicola,* which are characterized as having particularly high productivity and enzymatic activity. Furthermore, in comparison with the glucoamylase derived from other organisms, the *Rhizopus*-derived glucoamylase exhibits a strong action on starch and its enzymological and chemical properties including optimum pH are particularly suitable for the saccharification of cereal starch. Because of these features, the *Rhizopus*-derived glucoamylase is considered to be best suited for alcohol production using non-cooked or low-temperature cooked starch (*See,* U.S. Pat. No. 4,514,496 and 4,092,434). It has been noted that upon the incubation of corn starch with *Rhizopus* glucoamylase, was used in conjunction with *Rhizopus* alpha amylase, the starch degradation by glucoamylase was accelerated. While it is not intended that the present invention be limited to any particular mechanism or theory, it is believed that *Rhizopus* glucoamylase has a stronger degradation activity than *Aspergillus niger* glucoamylase preparations which also contain α-amylase (*See*, Yamamoto *et al.*, Denpun Kagaku, 37:129-136 [1990]). One commercial preparation that finds use in the present invention is the glucoamylase preparation derived from the Koji culture of a strain of *Rhizopus niveus* available from Shin Nippo Chemical Co., Ltd. Another commercial preparation that finds use in the present invention is the commercial starch hydrolyzing composition M1 is available from Biocon India (Bangalore, India).

As understood by those in the art, the quantity of glucoamylase used in the methods of the present invention depends on the enzymatic activity of the glucoamylase (*e.g.*, DISTILLASE® L-400). Generally, an amount between 0.001 and 2.0 ml of a solution of the glucoamylase is added to 450 gm of a slurry adjusted to 20-35% dry solids, the slurry being the liquefied mash during the saccharification and/or in the hydrolyzed starch and sugars during the fermentation. In some embodiments, the glucoamylase is added in an amount between 0.005 and 1.5 ml

of such a solution. In some preferred embodiments, the enzyme is added at an amount between 0.01 and 1.0 ml of such a solution.

As indicated above, pullulanases also find use in the methods of the present invention. These enzymes hydrolyze alpha.-1,6-glucosidic bonds. Thus, during the saccharification of the liquefied starch, pullulanases remove successive glucose units from the non-reducing ends of the starch. This enzyme is capable of hydrolyzing both the linear and branched glucosidic linkages of starch, amylose and amylopectin.

Additional enzymes that find use in the present invention include starch hydrolyzing (RSH) enzymes, including *Humicola* RSH glucoamylase enzyme preparation (*See*, U.S. Patent No. 4,618,579). This *Humicola* RSH enzyme preparation exhibits maximum activity within the pH range of 5.0 to 7.0 and particularly in the range of 5.5 to 6.0. In addition, this enzyme preparation exhibits maximum activity in the temperature range of 50° C to 60° C. Thus, in each of the steps of the present invention in which this enzyme is used, the enzymatic solubilization of starch is preferably carried out within these pH and temperature ranges.

In some embodiments, *Humicola* RSH enzyme preparations obtained from the fungal organism strain *Humicola grisea var. thermoidea* find use in the methods of the present invention. In some particularly preferred embodiments, these *Humicola* RSH enzymes are selected from the group consisting of ATCC (American Type Culture Collection) 16453, NRRL (USDA Northern Regional Research Laboratory) 15219, NRRL 15220, NRRL 15221, NRRL 15222, NRRL 15223, NRRL 15224, and NRRL 15225, as well as genetically altered strains derived from these enzymes.

Additional RSH glucoamylases that find use in the methods of the present invention include *Rhizopus* RSH glucoamylase enzyme preparations. In some embodiments, the enzyme obtained from the Koji strain of *Rhizopus niveus* available from Shin Nihon Chemical Co., Ltd., Ahjyo, Japan, under the tradename "CU CONC" is used. Another useful enzyme preparation is a commercial digestive from *Rhizopus* available from Amano Pharmaceutical under the tradename "GLUCZYME" (*See*, Takahashi *et al.*, J. Biochem., 98:663-671 [1985]). Additional

enzymes include three forms of glucoamylase (EC 3.2.1.3) of a *Rhizopus* sp., namely "Gluc1" (MW 74,000), "Gluc2" (MW 58,600) and "Gluc 3" (MW 61,400). Gluc1 was found to be 22-25 times more effective than Gluc2 or Gluc3. Thus, although Gluc2 and Gluc3 find use in the present invention, because Gluc1 tightly binds to starch and has an optimum pH of 4.5, Gluc1 finds particular use in the present invention. An additional RSH glucoamylase enzyme preparation for use in the present invention includes enzyme preparations sold under the designation "M1," available from Biocon India, Ltd., Bangalore, India. M1 is a multifaceted enzyme composition or mixture, as indicated by the high performance liquid chromatography spectra of Figure I and the SDS gel of Figure 2.

As noted above, in most embodiments, *Humicola* RSH glucoamylase enzyme preparations contain glucoamylase activity as well as a potentiating factor which solubilizes starch. The relative proportions of potentiating factor and glucoamylase activity in other RSH enzyme preparations may vary somewhat. However, with RSH glucoamylase enzyme preparations that find use in the present invention, there is usually ample potentiating factor produced along with the glucoamylase fraction. Accordingly, the activity of the RSH glucoamylase enzyme preparations is defined in terms of their glucoamylase activity.

Glucoamylase activity can also be measured for purposes of this invention in 10 D.E. units for either RSH enzyme preparation or conventional glucoamylase. A "10 D.E. unit" is the amount of either type of enzyme which produces 1 micromole of glucose per minute under the assay conditions. To determine glucoamylase activity for purposes of this invention, one-tenth ml of enzyme preparation, diluted if necessary, containing 0.06 units to 1.1 units is added to 0.9 ml of substrate solution preheated at 50°C for 5 minutes. The substrate solution consists of 40 parts by volume 0.25M sodium acetate buffer (pH 5.5) and 50 parts by volume 4% by weight 10 D.E. maltodextrin in water. The substrate solution is kept at 50°C for 5 minutes before the enzyme solution is added. After 10 minutes, the reaction is quenched by pouring into a preheated 16 mm test tube and heating in a 100°C water bath for 6 minutes. Glucose concentration is determined by any convenient method (*e.g.*, glucose reagent kit No. 15-UV from Sigma Chemical Co. or with an instrument such as the Technicon Autoanalyzer).

A particularly useful enzymatic composition includes a mixture of glucoamylase (*e.g.*, DISTILLASE®) and RSH (*e.g.*, M1). The amount of the glucoamylase useful in this combination is in the range of .2 to about 1.0 GAU units of glucoamylase per gram of granular solids. A more useful amount of glucoamylase is between about 0.75 to .5 GAU per gram of solids. The range of starch hydrolyzing enzyme (M1) present in this mixture ranges from 0.2 starch hydrolyzing units (RSHU) to about 1.0 RSHU per gram of solids. One particularly useful mixture includes about 0.6 GAU DISTILLASE® per gram of corn solids and 0.2 RSHU M1 per gram of corn solids.

In addition to the use of enzymatic compositions containing the above described enzymes, the present invention provides methods in which a microorganism is exposed to a substrate and uses the substrate to produce the desired end-product. Thus, in some embodiments, contacting the substrate or intermediate with a fungal, bacterial or other microorganism that produces the desired end-product is used to convert the substrate or intermediate to the desired intermediate or end-product.

In preferred embodiments of the present invention, once the carbon source is enzymatically converted to the intermediate, it is converted into the desired end-product by the appropriate methodology. Conversion is accomplished via any suitable method (*e.g.*, enzymatic or chemical). In one preferred embodiment, conversion is accomplished by bioconversion of the intermediate by contacting the intermediate with a microorganism. In alternate preferred embodiments, the respective substrate-converting enzyme and the intermediate-converting enzyme are placed in direct contact with the substrate and/or intermediate. In some embodiments, the enzyme(s) are provided as isolated, purified or concentrated preparations.

In further embodiments, the substrate and/or intermediate are placed in direct contact with a microorganism (*e.g.,* bacterium or fungus) that secretes or metabolizes the respective substrate or intermediate. Thus, the present invention provides means for the bioconversion of a substrate to an end-product. In some embodiments, at least one intermediate compound is produced during this conversion process.

In some embodiments, microorganisms that are genetically modified to express enzymes not normally produced by the wild-type organism are utilized. In some particularly preferred embodiments, the organisms are modified to overexpress enzymes that are normally produced by the wild-type organism.

Indeed, commercially available alpha-amylases and glucoamylases find use in the methods of the present invention in economically realistic enzyme concentrations. Although commonly used fermentation conditions do not utilize optimum temperatures, the pH conditions for fermentation do correspond closely to the optimum pH for commercially available saccharification enzymes (*i.e.*, the glucoamylases). In some embodiments of the present invention, complete saccharification to glucose is favored by the gradual solubilization of granular starch. Presumably, the enzyme is always exposed to low concentrations of dextrin. In addition, the removal of glucose throughout the fermentation maintains a low glucose content in the fermentation medium. Thus, glucoamylase is exposed to low concentration of glucose. In consequence, the glucoamylase is used so effectively that economically feasible dosage levels of glucoamylase are suitable for use in the methods of the present invention (*i.e.*, glucoamylase dosage of 0.05-10.0 GAU/g of starch; and preferably 0.2-2.0 GAU/g starch).

The dosages provided above for glucoamylase only approximate the effective concentration of the enzymatic saccharification activity in the fermentation broth, as an additional proportion of the saccharification activity is contributed by the alpha-amylase. Although it is not intended that the present invention be limited to any particular mechanism or theory, it is believed that the alpha-amylase further widens the holes bored by glucoamylase on starch granules (*See*, Yamamoto *et al., supra*). Typically, the use of commercially available alpha-amylases results in the production of significant amounts of sugars, such as glucose and maltose.

Addition of the alpha-amylase from *Aspergillus oryzae* (*e.g.*, FUNGAMYL) to wort has been suggested to the brewing industry. This particular enzyme saccharifies dextrins to maltotriose and maltose. Thus, although the purpose of the alpha-amylase is to liquefy the starch, its saccharification propensity also makes the alpha-amylase some part of the saccharifying enzyme content. It is believed that an alpha amylase is present in the M1 composition.

It is also contemplated that addition of the alpha-amylase from *Aspergillus oryzae* (*e.g.*, CLARASE® L (Genencor International Inc.) to wort will find use in the brewing industry. This particular enzyme saccharifies dextrins to maltotriose and maltose. Thus, although the purpose of the alpha-amylase is to liquefy starch, its saccharification propensity also make the alpha-amylase a portion of the saccharifying enzyme content.

Furthermore, some commercially available glucoamylases contain some alpha-amylase activity. Thus, it is possible (albeit usually not practical) to ferment particulate starch in the presence solely of glucoamylase. However, it is not intended that such embodiments be excluded from the present invention. Thus, it is also contemplated that commercially available starch hydrolyzing enzymes will find use in the present invention as part of a enzyme mixture which includes starch hydrolyzing enzymes, alpha amylases and glucoamylases. In most embodiments of the methods of the present invention, an effective amount of alpha-amylase is added to a slurry of particulate starch. Those of skill in the art understand that in addition to the uncertain amount of alpha-amylase activity contributed by glucoamylase, the effective activity of the alpha-amylase may be quite different from the unit activity values given by the supplier. The activity of alpha-amylase is pH dependent, and may be different at the pH range selected for the fermentation (*i.e.*, as compared with the test conditions employed by the suppliers for their reported unit activity values). Thus, some preliminary experiments are contemplated as being sometimes necessary in order establish the most effective dosages for alpha-amylases, including those not explicitly described herein, but find use in the methods of the present invention.

In some most preferred embodiments, the alpha-amylase dosage range for fungal alpha-amylases is from 0.02 GAU/g (Fungal Amylase Units) to 2.0 FAU/g of starch, although in some particularly preferably embodiments, the range is 0.05-0.6 FAU/g. One "FAU" is the amount of enzyme which breaks down 5260 mg of starch per hour under a standardized set of conditions, and corresponds to approximately 25 SKB units (*See*, Cerial Chem.,, 16:712-723 [1939]). In most embodiments utilizing *Bacillus* alpha-amylases, the range is 0.01 KNU/g to 0.6 KNU/g, preferably 0.05 to 0.15 KNU/g, the NU (or Novo Unit) being the amount of enzyme which

breaks down 5.26 mg of starch per hour under a standardized set of conditions. One KNU corresponds to 1000 NU.

It is contemplated that the uncertainty as to the real activity of both the glucoamylase and the alpha-amylase in the fermenting slurry will require some preliminary investigation into the practice of some embodiments. Optimization considerations include the fact that increasing the alpha-amylase dosage with a constant glucoamylase content, increases the fermentation rate. In addition, increasing the glucoamylase dosage with a constant alpha-amylase content increases the fermentation rate. Holding the dosage of enzyme constant and/or increasing the starch content in the slurry also increase the fermentation rate. Indeed, it is contemplated that in some embodiments, the optimum alpha-amylase dosage well exceeds dosages heretofore recommended for liquefying starch; the optimum glucoamylase may well exceed dosages recommended for saccharifying syrups. However, enzyme dosage levels should not be confused with enzyme usage. Substantial proportions of the enzymes dosed into the starch slurry are available for recovery from the fermentation broth for use anew to ferment granular starch.

A further consideration arising from employment of the enzymes at fermentation temperatures is that although the enzymes exhibit low relative activity (*e.g.*, activity of the alpha-amylase from *B. licheniformis* at fermentation temperatures is not more than about 25% of maximum activity), the low relative activity is counterbalanced by the extended duration of the 48-120 hours of fermentation, and by the extended half-life of enzymes that have not been subjected to elevated temperatures. Indeed, it has been determined that more than 90% of the enzyme activity remains after 72 hours of fermentation. It was also noted that the use of M1 resulted in at least 50% of the starch solids being hydrolyzed after 72 hours, at least 90% hydrolyzed after 72 hours and in some cases, at least 95% hydrolyzed after 72 hours.

The alpha-amylase of *B. licheniformis* (SPEZYME® AA or SPEZYME® FRED enzymes; Genencor) is sufficiently stable to withstand brief exposures to still pot temperatures. Thus, recycle of stillage can be used as a way to recycle alpha-amylase. However, recovery of enzyme in recycled stillage requires care, in

avoiding subjecting the fermentation broth to ethanol stripping temperatures that deactivate the enzyme(s). For example, the alcohol may be vacuum stripped from the fermentation broth and such stillage recycled to recover the enzymes suitable for use in subsequent reactions.

However, as earlier described, some RSHs (e.g., the enzyme obtained from *Rhizopus*) are available that convert starch to glucose at non-cooking temperatures (*e.g.*, 25 to 35 °C), reducing the need for exposing the enzymatic composition to still pot temperatures. This reduces the energy costs of converting the carbon substrate to the desired end-product, thereby reducing the overall costs of manufacturing. Thus, these enzymes find particular use in the methods of the present invention.

In preferred embodiments of the present invention, once the carbon source is enzymatically converted to the intermediate, it is converted into the desired end-product by the appropriate methodology. Conversion is accomplished via any suitable method (*e.g.*, enzymatic or chemical). In one preferred embodiment, conversion is accomplished by bioconversion of the intermediate by contacting the intermediate with a microorganism. In alternate preferred embodiments, the respective substrate-converting enzyme and the intermediate-converting enzyme are placed in direct contact with the substrate and/or intermediate. In some embodiments, the enzyme(s) are provided as isolated, purified or concentrated preparations.

The desired end-product can be any product that may be produced by the enzymatic conversion of the substrate to the end-product. In some preferred embodiments, the substrate is first converted to at least one intermediate and then converted from the intermediate to an end-product. For example, hexoses can be bioconverted into numerous products, such as ascorbic acid intermediates, ethanol, 1,3-propanediol, and gluconic acid. Ascorbic acid intermediates include but are not limited to 2,5-diketogluconate, 2 KLG (2-keto-L-gluconate), and 5-KDG (5-keto-D-gluconate). Gluconate can be converted from glucose by contacting glucose with glucose dehydrogenase (GDH). In addition, gluconate itself can be converted to 2-KDG (2-keto-D-gluconate) by contacting gluconate with GDH. Furthermore, 2-KDG can be converted to 2,5-DKG by contacting 2-KDG with 2-KDGH. Gluconate can also be converted to 2-KDG by

contacting gluconate with 2KR. Glucose can also be converted to 1,3-propanediol by contacting glucose with *E. coli*. In addition, glucose can be converted to succinic acid by contacting glucose with *E. coli*.

Additional embodiments, as described herein are also provided by the present invention. In one particularly preferred embodiment of the present invention, the end-product is ethanol. In some embodiments in which glucose is an intermediate, it is converted to ethanol by contacting glucose with an ethanologenic microorganism. In contacting the intermediate with an intermediate converting enzyme, it is contemplated that isolated and/purified enzymes are placed into contact with the intermediate. In yet another embodiment, the intermediate is contacted with bioconverting agents such as bacteria, fungi or other organism that takes in the intermediate and produces the desired end-product. In some embodiments, the organism is wild-type, while in other embodiments it is mutated.

Preferred examples of ethanologenic microorganisms include ethanologenic bacteria expressing alcohol dehydrogenase and pyruvate decarboxylase, such as can be obtained with or from *Zymomonas mobilis* (*See e.g.*, U.S. Pat. Nos. 5,028,539, 5,000,000, 5,424,202, 5,487,989, 5,482,846, 5,554,520, 5,514,583, and copending applications having U.S. Ser. No. 08/363,868 filed on Dec. 27, 1994, U.S. Ser. No. 08/475,925 filed on Jun. 7, 1995, and U.S. Ser. No. 08/218,914 filed on Mar. 28, 1994, the teachings of all of which are hereby incorporated by reference, in their entirety).

In additional embodiments, the ethanologenic microorganism expresses xylose reductase and xylitol dehydrogenase, enzymes that convert xylose to xylulose. In further embodiments, xylose isomerase is used to convert xylose to xylulose. In additional embodiments, the ethanologenic microorganism also expresses xylulokinase, an enzyme that catalyzes the conversion of xylulose to xylulose-5-phosphate. Additional enzymes involved in the completion of the pathway include transaldolase and transketolase. These enzymes can be obtained or derived from *Escherichia coli, Klebsiella oxytoca* and *Erwinia* species (*See e.g.*, U.S. Pat. No. No. 5,514,583).

In some particularly preferred embodiments, a microorganism capable of fermenting both pentoses and hexoses to ethanol are utilized. For example in

some embodiments, a recombinant organism which inherently possesses one set of enzymes and which is genetically engineered to contain a complementing set of enzymes is used (*See e.g.*, U.S. Pat. Nos. 5,000,000, 5,028,539, 5,424,202, 5,482,846, 5,514,583, and WO 95/13362). In some embodiments, particularly preferred microorganisms include *Klebsiella oxytoca* P2 and *E. coli* KO11.

In some embodiments, supplements are added to the nutrient medium (*i.e.*, the culture medium), including, but not limited to vitamins, macronutrients, and micronutrients. Vitamins include, but are not limited to choline chloride, nicotinic acid, thiamine HCl, cyanocobalamin, p-aminobenzoic acid, biotin, calcium pantothenate, folic acid, pyridoxine.HCl, and riboflavin. Macronutrients include, but are not limited to $(NH_4)_2SO_4$, K_2HPO_4, NaCl, and $MgSO_4$. $7H_2O$. Micronutrients include, but are not limited to $FeCl_3$ $6H_2O$, $ZnCl_2.4H_2O$, $CoCl_2.6H_2O$, molybdic acid (tech), $CuCl_3.2H_2O$, $CaCl_2.2H_2O$, and H_3BO_3.

Media and Carbon Substrates

The conversion media in the present invention must contain suitable carbon substrates. Suitable carbon substrates include, but are not limited to biomass, monosaccharides (*e.g.*, glucose and fructose), disaccharides (*e.g.*, lactose and sucrose), oligosaccharides, polysaccharides (*e.g.*, starch and cellulose), as well as mixtures thereof, and unpurified mixtures from renewable feedstocks such as cheese whey permeate, cornsteep liquor, sugar beet molasses, and barley malt. In additional embodiments, the carbon substrate comprises one-carbon substrates such as carbon dioxide, or methanol for which metabolic conversion into key biochemical intermediates has been demonstrated.

Although it is contemplated that all of the above mentioned carbon substrates and mixtures thereof will find use in the methods of the present invention, preferred carbon substrates include monosaccharides, disaccharides, oligosaccharides, polysaccharides, and one-carbon substrates. In more particularly preferred embodiments, the carbon substrates are selected from the group consisting of glucose, fructose, sucrose and single carbon substrates such as methanol and carbon dioxide. In a most particularly preferred embodiment, the substrate is glucose.

As known in the art, in addition to an appropriate carbon source, fermentation media must contain suitable nitrogen source(s), minerals, salts, cofactors, buffers and other components suitable for the growth of the cultures and promotion of the enzymatic pathway necessary for the production of the desired end-product (*e.g.*, glycerol). In some embodiments, (II) salts and/or vitamin B_{12} or precursors thereof find use in the present invention.

Culture Conditions

Typically, cells are grown at approximately 30 °C. in appropriate media. Preferred growth media utilized in the present invention include common commercially prepared media such as Luria Bertani (LB) broth, Sabouraud Dextrose (SD) broth or Yeast Malt Extract (YM) broth. However, other defined or synthetic growth media may also be used, as appropriate. Appropriate culture conditions are well-known to those in the art.

In some embodiments, agents known to modulate catabolite repression directly or indirectly (*e.g.*, cyclic adenosine 2':3'-monophosphate or cyclic adenosine 2':5'-monophosphate), are incorporated into the reaction media. Similarly, the use of agents known to modulate enzymatic activities (*e.g.*, sulphites, bisulphites and alkalis) that lead to enhancement of glycerol production also find use in conjunction with or as an alternative to genetic manipulations.

Suitable pH ranges for fermentation are between pH 5.0 to pH 9.0; while the range of pH 6.0 to pH 8.0 is particularly preferred for the initial conditions of the reaction system. Furthermore, reactions may be performed under aerobic, microaerophilic, or anaerobic conditions, as suited for the organism utilized.

Batch and Continuous Fermentations

In some preferred embodiments, the present process uses a batch method of fermentation. A classical batch fermentation is a closed system, wherein the composition of the media is set at the beginning of the fermentation and is not subject to artificial alterations during the fermentation. Thus, at the beginning of the fermentation the medium is inoculated with the desired organism(s). In this method, fermentation is permitted to occur without the addition of any components to the

system. Typically, a batch fermentation qualifies as a "batch" with respect to the addition of the carbon source and attempts are often made at controlling factors such as pH and oxygen concentration. The metabolite and biomass compositions of the batch system change constantly up to the time the fermentation is stopped. Within batch cultures, cells moderate through a static lag phase to a high growth log phase and finally to a stationary phase where growth rate is diminished or halted. If untreated, cells in the stationary phase eventually die. In general, cells in log phase are responsible for the bulk of production of end product or intermediate.

A variation on the standard batch system is the "fed-batch fermentation" system, which also finds use with the present invention. In this variation of a typical batch system, the substrate is added in increments as the fermentation progresses. Fed-batch systems are useful when catabolite repression is apt to inhibit the metabolism of the cells and where it is desirable to have limited amounts of substrate in the media. Measurement of the actual substrate concentration in fed-batch systems is difficult and is therefore estimated on the basis of the changes of measurable factors such as pH, dissolved oxygen and the partial pressure of waste gases such as CO_2. Batch and fed-batch fermentations are common and well known in the art.

It is also contemplated that the methods of the present invention are adaptable to continuous fermentation methods. Continuous fermentation is an open system where a defined fermentation media is added continuously to a bioreactor and an equal amount of conditioned media is removed simultaneously for processing. Continuous fermentation generally maintains the cultures at a constant high density where cells are primarily in log phase growth.

Continuous fermentation allows for the modulation of one factor or any number of factors that affect cell growth and/or end product concentration. For example, in one embodiment, a limiting nutrient such as the carbon source or nitrogen level is maintained at a fixed rate an all other parameters are allowed to moderate. In other systems, a number of factors affecting growth can be altered continuously while the cell concentration, measured by media turbidity, is kept constant. Continuous systems strive to maintain steady state growth conditions. Thus, cell loss due to media being drawn off must be balanced against the cell

growth rate in the fermentation. Methods of modulating nutrients and growth factors for continuous fermentation processes as well as techniques for maximizing the rate of product formation are well known in the art of industrial microbiology.

In some embodiments, the present invention is practiced using batch processes, while in other embodiments, fed-batch or continuous processes, as well as any other suitable m0de of fermentation are used. Additionally, in some embodiments, cells are immobilized on a substrate as whole-cell catalysts and are subjected to fermentation conditions for the appropriate end-product production.

Identification and Purification of the End-Product

Methods for the purification of the end-product from fermentation media are known in the art. For example, propanediols can be obtained from cell media by subjecting the reaction mixture to extraction with an organic solvent, distillation and column chromatography (*See e.g.*, U.S. Pat. No. 5,356,812). A particularly good organic solvent for this process is cyclohexane (*See*, U.S. Pat. No. 5,008,473).

In some embodiments, the end-product is identified directly by submitting the media to high pressure liquid chromatography (HPLC) analysis. One method of the present invention involves analysis of fermentation media on an analytical ion exchange column using a mobile phase of 0.01 N sulfuric acid in an isocratic fashion.

Identification and Purification of the Enzymes

The enzyme levels in the media can be measured by enzyme assays. For example in the manufacture of 1,3-propanediol, the levels of expression of the proteins G3PDH and G3P phosphatase are measured by enzyme assays. The G3PDH activity assay relies on the spectral properties of the cosubstrate, NADH, in the DHAP conversion to G-3-P. NADH has intrinsic UV/vis absorption and its consumption can be monitored spectrophotometrically at 340 nm. G3P phosphatase activity can be measured by any method of measuring the inorganic phosphate liberated in the reaction. The most commonly used detection method used the visible spectroscopic determination of a blue-colored phosphomolybdate ammonium complex.

Thus, although there are various superficial resemblances between the methods known in the art and the methods of the present invention, the present invention provides more comprehensive objectives that are reflected in a great number of detail features believed to be unique to practice of this invention, including notably enzyme recycling, biomass and starch recycling.

Recovery

Overall, recovery of enzymes in recycled stillage requires care, in order to avoid subjecting the conversion media to temperatures that deactivate the enzymes. In one example, for the recovery of ethanol, the alcohol is vacuum stripped from the fermentation broth and the stillage is recycled, in order to recover the enzymes. In embodiment, enzymes are recovered through the use of ultrafiltration or an electrodialysis device and recycled.

Process Considerations

As indicated above, fermentation of granular starch slurry has completely different characteristics than fermentation of a syrup. Generally, a concentration of about 20% solids in solution is considered the maximum sugar content in a fermentation medium, as higher concentrations create difficulties at the onset and at the end of fermentation. However, no similar limits exist in the fermentation of a starch slurry. The concentration of starch in the slurry may vary from 10 -35 %, with no discernable consequences at the onset of fermentation. Increasing starch concentration (*e.g.*, at constant enzyme dosages) speeds up the bioconversion rate, or conversely, allows for lowering the enzyme dosages required to achieve a given bioconversion rate. In any event fermenting until the broth has 7-10% alcohol, as is prevalent in the fermentation arts, is still possible. The excess (*i.e.*, residual) granular starch may be recovered, along with substantial amounts of enzymes and subjected to renewed fermentation. Thus, control over starch concentration is a major process parameter for practice of this invention.

In one preferred embodiment, means for bioconversion and fermentation of a granular starch slurry having 10-35% starch by weight are provided. In some preferred embodiments, fermentation of a 10-35% starch slurry with *E. coli* results

in the production of residual starch when fermentation has proceeded to the intended organic acid or 1,3-propane diol content levels. However, this reaction is dependent on the microorganism and bioprocessing conditions used and, therefore, recycling of the enzymes on the starch particles occurs when the residual starch is again fermented. However, even when a 25-35% starch slurry is fermented, in preferred embodiments, the fermentation is halted before complete disappearance of the granular starch, for fermentation anew. Thus, recycling of starch is a facile way to recover enzymes for reuse.

In an alternative embodiment, means for fermentation of a granular starch slurry of 25-25% by weight are provided. Fermenting a 25-35% starch slurry with common baker's yeast will invariably result in residual starch when fermentation has proceeded to the intended alcohol content levels (*e.g.*, 7-10%), dependent on the microorganism used and the recycling of the enzymes on the starch particles occurs when the residual starch is again fermented. However, it is not intended that the present invention be limited to this range, as other weight percentages will find use in the present invention, depending upon the substrate and/or enzyme system utilized in the methods. For example, in some embodiments, a granular starch slurry of 10-35% by weight is preferred. A particularly useful microorganism is one that is resistant to the alcohol produced by the process.

In one preferred embodiment of the present invention, the (granular) starch and microorganisms are removed together (*e.g.*, by centrifugation or filtration). This removed starch and microorganisms are mixed with fresh granular starch and additional aliquot(s) of enzyme(s) as needed, to produce a fermentation charge for another fermentation run.

In another embodiment, bioconversion and fermentation of a corn-stover slurry having 10-35% cellulosics by weight is provided. In one embodiment, fermenting a 10-35% cellulosic slurry with *E. coli* results in residual cellulosic when fermentation has proceeded to the intended organic acid or 1,3-propane diol content levels. This reaction is dependent upon the microorganism and bioprocessing conditions used. As above, recycling of the enzymes on the cellulosics occurs when the residual corn-stover is again fermented. However, even when a 25-35% cellulosics slurry is fermented, in some preferred

embodiments, the fermentation is halted before the complete disappearance of the stover, for fermentation anew. Thus, recycling stover is a facile way to recover enzymes for reuse.

In yet another preferred embodiment, the granular starch or corn stover and microorganisms are removed together (*e.g.*, by centrifugation or filtration). This mixture of removed granular starch or corn stover and microorganisms is mixed with fresh granular starch or corn stover and additional aliquot(s) of enzyme(s) as needed, to produce a fermentation charge for another fermentation run.

As recognized by those of skill in the art, engineering trade-offs are contemplated in arriving at optimum process details; these trade-offs are contemplated to vary, depending upon each particular situation. Nonetheless, the methods provided herein provide the necessary teachings to make such trade-offs to obtain optimum processes. For example, to achieve the most rapid fermentation reasonable, high starch or cellulosic content, and high enzymes dosage are indicated. But, the consequential rapid fermentation tails off into generation of a level of nutrients in the fermentation broth, when then dictates recovery of the nutrients, or, alternatively that fermentation be halted at a relatively low end-product (*e.g.,* alcohol) content. However, in situations where relatively low fermentation rates are acceptable, then (with high starch content slurries) enzyme dosage is relatively low and nutrient losses are held to levels heretofore accepted by the fermentation arts. In cases where maximum yield of end-product (*e.g.*, alcohol) is a principal objective, then low starch content slurries, moderate alpha-amylase dosage, and high glucoamylase dosage find use in the present invention. However, it is not intended that the present invention be limited to any particular method design.

As indicated herein, the present invention saves considerable thermal energy. However, just as the starting substrate (*e.g.*, starch) is never subjected to the thermal conditions used for liquefactions, the substrate is not thermally sterilized. Thus, it is contemplated that is some embodiments, the starting substrate (*e.g.*, granular starch) adds contaminating microorganisms to the fermentation medium. Thus, in some embodiments, it is advantageous to seed the fermentation medium with a large number of the product-producing microorganisms

that are associated with recycled substrate (*e.g.*, starch). By greatly outnumbering the contaminants, the recycled microorganisms overwhelm any contaminating microorganisms, thereby dominating the fermentation, resulting in the production of the desired end-product. Thus, in some embodiments, the method involves seeding the fermentation medium with the great number of the ethanol producing microorganism that are likely to accompany the recycled granular starch. Through their great numbers, the recycled microorganisms overwhelm any contaminating microorganisms, thereby dominating the fermentation, as is, of course, desired.

In some embodiments, the quantities of yeast initially charged into the fermentation vat may be in accord with prior art practices for ethanol fermentation, and can vary widely since the yeast cells will multiply during the course of the fermentation. Recycling of yeast cells is not necessary, although may be performed. In some embodiments, the yeast is removed from the residual starch particles prior to recycling of the residual starch. However, it is noted once again that practice of the present invention does not necessarily require a thermal treatment of the starch (*i.e.*, thermal conditions that would heat sterilize the starch). Thus, as with bacteria, it is advisable in some embodiments of the present invention to charge relatively large proportions of yeast cells into the fermentation in order to help overcome the likelihood of (inadvertent) contamination. In addition, in some embodiments, antimicrobials are added to the fermentation medium to suppress growth of contaminating microorganisms. In further embodiments, cold sterilization techniques are utilized with the materials involved in the methods.

In most preferred embodiments, the practice of the present invention controls the fermentation rate by releasing metabolizable sugars to the microorganisms (*e.g.*, yeast) at a controlled rate and maintaining the concentration of the intermediate (*e.g.*, glucose) at a level that does not trigger enzyme inhibition or catabolite repression. This approach is very different from what was done prior to the development of the present invention. Indeed, the prior art suggests treating solid starch with enzymes prior to fermentation and/or including enzymes in the fermentation medium to conserve energy and/or to improve fermentation efficiency. However, these teachings do not alter the character of the fermentation so as to avoid the adverse effects of catabolite repression and/or enzymatic inhibition. The

present invention also provide means to counter the adverse effects of producing undesired by-products from glucose. The present invention also provides means to conserve energy, particularly in comparison with prior art methods involving high temperature starch liquefaction. Indeed, the present invention provides means to conserve more thermal energy than other methods. The present invention provides methods that operate with high fermentation efficiency, in part because product losses due to starch retrogradation, incomplete saccharification, and incomplete fermentation of fermentables are reduced. The ability to tailor the fermentation rate through control of starch concentration and enzyme content and proportions includes the capability of creating a fermentation broth product with minimal carbohydrate content.

As indicated above, in some embodiments, the quantities of microorganisms and enzymes initially charged into the fermentation vat or bioreactor are in accord with prior art practices for the fermentation or bioconversion of various products. These quantities will vary, as the microbial cells multiply during the course of the fermentation whereas enzymes used for bioconversion will have a limited half-life. Although in some embodiments, recycling of microorganisms is utilized, in many embodiments, it is not required for the practice of the present invention. In contrast, in particularly preferred embodiments, it is desirable to recycle enzymes (although it is not intended that the present invention be limited to methods which require the recycling of enzymes).

Thus, in some embodiments, the microbes are removed from the residual starch or biomass particles prior to recycling of the residual starch or biomass. However, it is again noted that practice of the present invention does not necessarily require thermal treatment of the starting substrate (*e.g.*, starch). Thus, in some embodiments, the starting substrate is heat-sterilized, while in other embodiments, it is not. Therefore, in some embodiments, the fermentation/bioconversion is conducted in the presence of a relatively large proportion of microorganisms, in order to overcome the effects of any contamination. In alternative embodiments, antimicrobials are added to the fermentation medium to suppress growth of contaminating microorganisms. In additional embodiments, cold sterilization techniques, UV radiation, 65°C

pasteurization are used to sterilize the starting (*e.g.,* substrate) materials. However, biomass poses no problem regarding sterilization of fermentation vats or bioreactors.

Use of starch as the starting material does not only address the above shortcomings of currently used methods, but has three additional significant benefits in terms of the raw material cost of corn starch vs. D-glucose, reduction of substrate and/or product based inhibition of enzymes employed in the bioconversion, and a concomitant significant reduction in the requirement of high enzyme dosages.

Various other examples and modifications of the description and Examples are apparent to a person skilled in the art after reading the disclosure without departing from the spirit and scope of the invention; it is intended that all such examples or modifications be included within the scope of the appended claims. All publications and patents referenced herein are hereby incorporated by reference in their entirety.

EXPERIMENTAL

The following examples are provided in order to demonstrate and further illustrate certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof. Indeed, it is contemplated that these teachings will find use in further optimizing the process systems described herein.

In the experimental disclosure which follows, the following abbreviations apply: wt% (weight percent); °C (degrees Centigrade); rpm (revolutions per minute); H_2O (water); dH_2O (deionized water); (HCl (hydrochloric acid); aa (amino acid); bp (base pair); kb (kilobase pair); kD (kilodaltons); gm (grams); µg (micrograms); mg (milligrams); ng (nanograms); µl (microliters); ml (milliliters); mm (millimeters); nm (nanometers); µm (micrometer); M (molar); mM (millimolar); µM (micromolar); U (units); V (volts); MW (molecular weight); psi (pounds per square inch); sec (seconds); min(s) (minute/minutes); hr(s) (hour/hours); Q.S. and q.s. (quantity sufficient); OD (optical density); OD_{280} (optical density at 280 nm); OD_{600} (optical density at 600 nm); PAGE (polyacrylamide gel electrophoresis); PBS (phosphate

buffered saline [150 mM NaCl, 10 mM sodium phosphate buffer, pH 7.2]); Cerestar (Cerestar, a Cargill, inc., company, Minneapolis, MN); SDS (sodium dodecyl sulfate); Tris (tris(hydroxymethyl)aminomethane); w/v (weight to volume); v/v (volume to volume); ATCC (American Type Culture Collection, Rockville, MD); Difco (Difco Laboratories, Detroit, MI); GIBCO BRL or Gibco BRL (Life Technologies, Inc., Gaithersburg, MD); Genencor (Genencor International, Inc., Palo Alto, CA); Shin Nihon (Shin Nihon, Japan); BioRad (BioRad Laboratories, Hercules, CA); and LeSaffre (LeSaffre Yeast Corporation, Milwaukee, WI).

In the following Examples, additional various media and buffers known to those in the art were used, including the following:

EXAMPLE 1

Fermentation of Non-Cooked Corn Mash

In this Example, experiments conducted to compare starch hydrolyzing enzyme activity with a glucoamylase on uncooked starch are described.

Fermentation experiments were carried out in 250 ml flasks that were incubated in a 30°C shaker water bath. For this experiment 112 gm of 32.1% ground corn slurry containing 0.5% dry corn steep was placed in 250 ml flasks. The pH of the slurry was about 5.7, which required no further adjustment. The desired enzymes (DISTILLASE® or Sumizyme CU; Shin Nihon) were added, along with 0.37 gm of Red Star active dry yeast (LeSaffre) to start the saccharification and fermentation. During the fermentation a sample of the beer was centrifuged and .5 ml of supernatant was added to a test tube containing .05 ml of 1.1 N H_2SO_4 containing 5% glutaraldehyde to terminate both the fermentation and enzyme action. The sample was then diluted with 5.0 ml water and then subjected to HPLC analysis on Bio Rad HPX-87H column. The results are shown in Table 1 below.

Table 1.

Fermentation of Uncooked Ground Corn Comparing Sumizyme CU With Distillase

Fermentation at 30°C With enzyme dosage as GAU per gm of corn.

Flask	Enzyme	Hours	% W/V % DP>2	% W/V DP-2	% W/V DP-1	% W/V Lactic	% W/V Glycerol	% V/V Ethanol
1	0.20 GAU/g CU	24	0.27	0	0.02	0.12	0.66	8.91
		48	0.29	0	0	0.06	0.71	13.68
		72	0.30	0	0	0.02	0.59	15.07
2	0.20 GAU/g Dist	24	0.25	0	0	0.13	0.28	3.05
		48	0.20	0	0	0.37	0.26	4.59
		72	0.19	0	0	0.49	0.24	6.10
3	0.40 GAU/g Dist	24	0.20	0	0	0.09	0.34	4.59
		48	0.21	0	0	0.12	0.38	7.24
		72	0.17	0	0	0.11	0.38	9.45
4	0.75 GAU/g Dist	24	0.22	0	0	0.08	0.40	5.51
		48	0.20	0	0	0.06	0.44	9.54
		72	0.21	0	0	0.03	0.45	12.13
5	1.00 GAU/g Dist	24	0.24	0	0	0.11	0.49	6.38
		48	0.22	0	0	0.13	0.41	10.64
		72	0.18	0	0	0.07	0.61	13.29

CU = Sumizyme CU from Shin Nihon
Dist = Distillase L-400

As indicated in the Table 1, little if any detectable glucose is found in the beer, which indicated as the starch is being hydrolyzed it quickly was converted to ethanol by the yeast. Figure 1 provides a graph showing the ethanol content of the various tests.

These results could show that a starch hydrolyzing enzyme could convert the uncooked starch much more efficiently than DISTILLASE®. The rate of fermentation seems more related to the RSU activity. The .2 GAU/gm level of CU corresponds to .590 RHU/gm, while the 1.0 GAU/gm level of DISTILLASE® corresponds to only .108 RHU/gm. The RHU/GAU ratio for DISTILLASE® is .54 whereas the RHU/GAU ratio for CU is 2.98, which shows an enzyme with a high RHU/GAU ratio can better hydrolyze uncooked starch.

EXAMPLE 2

Fermentation of Ground Corn Slurry

In these experiments, the same procedure was used for this experiment as in Example 1, except that 35.9 % ground corn slurry was used (instead of corn mash), and prior to starting the fermentation the slurry was placed in a 65°C water for one

hour as a pasteurization step. No observed gelatinization of the slurry was observed. The enzymes tested were Sumizyme CU (Example 1), a *Rhizopus* glucoamylase preparation (M1) from Biocon assayed at 178 GAU/gm and 277 RHU/gm, and DISTILLASE® L-400 (Dist.) at 361 GAU/gm and 196 RHU/gm. Table 2 provides the conditions used for this study, and also summarizes the results.

Table 2.

Fermentation Uncooked Starch With Separate And Enzyme Combinations

Sample	Enzyme	Level	Enzme	Level	Hours	% W/V DP>2	% W/V DP-2	% W/V DP-1	% W/V Lactic	% W/V Glycerol	% V/V Ethanol
1	M1	.20 GAU/g			24	0.40	0.04	0.02	0.17	0.67	8.66
1					48	0.35	0.02	0.00	0.10	0.74	12.28
1					72	0.36	0.01	0.00	0.04	0.76	14.02
2	M1	.50 GAU/g			24	0.42	0.03	0.01	0.13	0.80	11.91
2					48	0.41	0.04	0.00	0.04	0.82	15.24
2					72	0.54	0.03	0.00	0.02	0.84	15.23
3	M1	.75 GAU/g			24	0.42	0.03	0.01	0.12	0.86	12.43
3					48	0.53	0.02	0.01	0.06	0.91	15.30
3					72	0.55	0.03	0.01	0.03	0.94	15.43
4	CU	.20 GAU/g			24	0.34	0.03	0.10	0.14	0.92	10.59
4					48	0.35	0.07	0.05	0.09	1.03	14.96
4					72	0.40	0.04	0.04	0.04	1.04	15.63
5	CU	.50 GAU/g			24	0.37	0.13	0.80	0.13	0.96	12.20
5					48	0.45	0.24	1.15	0.08	1.05	14.96
5					72	0.45	0.25	1.46	0.07	1.08	14.96
6	CU	.75 GAU/g			24	0.43	0.16	1.15	0.13	0.97	12.69
6					48	0.51	0.30	2.19	0.08	1.05	14.90
6					72	0.51	0.33	2.67	0.07	1.08	14.83
7	M1	.20 GAU/g	Dist	.2 GAU/g	24	0.41	0.04	0.02	0.14	0.71	9.19
7					48	0.35	0.01	0.00	0.07	0.75	13.06
7					72	0.40	0.02	0.00	0.02	0.78	15.20
8	M1	.20 GAU/g	Dist	.6 GAU/g	24	0.33	0.04	0.03	0.15	0.77	9.56
8					48	0.39	0.02	0.00	0.09	0.84	13.56
8					72	0.38	0.03	0.00	0.04	0.86	15.02
9	M1	.20 GAU/g	Dist	2.0 GAU/g	24	0.30	0.03	0.03	0.13	0.82	10.46
9					48	0.33	0.02	0.01	0.08	0.89	14.66
9					72	0.38	0.03	0.01	0.03	0.90	15.74

The ethanol results from the fermentations with M1 and CU are plotted in Figure 2A and 2B. At the .2 GAU/gm level for M1 the rate and yield of ethanol is less than the .5 and .75 levels indicating the .2 level is enzyme limiting. The .5 and .75 levels of M1 seem to give very similar results indicating that enzyme is no longer limiting. The results from CU similarly shows that the .2 enzyme level is somewhat limiting the fermentation, but is faster than .2 GAU/gm for M1 results. This indicates that the RHU activity is a better parameter that indicates the hydrolysis of uncooked starch. CU has about twice the RHU activity per GAU as does M1, and CU is seen to hydrolyze the uncooked starch faster at similar GAU

levels. At the .5 and .75 GAU/gm dosage excess glucose is observed particularly at the higher enzyme level. Actually it appears that starch hydrolyzing rate is faster than the fermentation rate. These results also show that at around 15% ethanol, the ethanol seems to become toxic to the yeast since the fermentations appeared to stop.

The graph provided at Figure 2, Panel C shows the ethanol results from the fermentations where M1 was added to DISTILLASE®. As the results show, adding DISTILLASE® to a low level of M1, .2 GAU/gm, both the rate and yield of ethanol increased improving the performance of M1. These results show that by adding a glucoamylase preparation with a GSH ratio greater than 1.5 to DISTILLASE®, which has only a GSH ratio less than .6, can hydrolyze uncooked starch so that ethanol can be made by a process that eliminates the cooking step.

EXAMPLE 3

Comparison of Cooked and Uncooked Corn Mash

In these experiments, fermentations were conducted similar to that described in Example 1, except 83 gm of 28.9% ground corn slurry were placed in 250 ml bottles containing a magnetic bar. The bottles were placed on a submersion magnetic stirrer in a 30°C water bath so that the mash was gently mixed during the fermentation. Combinations of DISTILLASE® and M1 were tested as shown in Table 3. These fermentation were started with .27 gm of dry yeast. After the fermentation, the beer was dried in a 65°C forced air oven to obtain what was considered the DDGS (Distillers Dry Grains plus the Solubles). In this manner a quantitative estimate of the DDGS was obtained, and the starch contend of the DDGS was obtained by a starch analysis technique. The HPLC profiles of the fermentations are also shown in Table 3.

Table 3.

Trial	M1 GAU/g	Dist. GAU/g	Hours	HPLC Profile % W/V DP>2	% W/V DP-2	% W/V DP-1	% W/V Lactic	% W/V Glycerol	% V/V Ethanol
1	0.10	0.00	24	0.23	0	0	0.08	0.28	3.40
1			48	0.26	0	0	0.46	0.56	5.66
1			72	0.27	0	0	0.62	0.33	6.95
2	0.10	0.20	24	0.24	0	0	0.11	0.41	5.70
2			48	0.27	0	0	0.09	0.46	9.21
2			72	0.32	0	0	0.04	0.48	11.44
3	0.10	0.40	24	0.19	0	0	0.14	0.52	6.87
3			48	0.26	0	0	0.12	0.61	10.87
3			72	0.27	0	0	0.05	0.63	12.98
4	0.10	0.60	24	0.22	0	0	0.15	0.59	7.79
4			48	0.26	0	0	0.12	0.67	11.93
4			72	0.29	0	0	0.05	0.69	13.63
5	0.10	1.00	24	0.18	0	0.01	0.15	0.69	9.07
5			48	0.23	0	0	0.10	0.76	12.74
5			72	0.29	0	0	0.05	0.79	14.14
6	0.20	0.00	24	0.22	0	0	0.12	0.42	5.64
6			48	0.27	0	0	0.08	0.43	8.86
6			72	0.32	0	0	0.03	0.46	11.33
7	0.20	0.20	24	0.23	0	0	0.15	0.54	7.29
7			48	0.25	0	0	0.13	0.53	11.15
7			72	0.22	0	0	0.07	0.66	13.09
8	0.20	0.40	24	0.21	0	0	0.15	0.62	8.46
8			48	0.25	0	0	0.13	0.70	12.38
8			72	0.27	0	0	0.06	0.53	13.65
9	0.20	0.60	24	0.25	0	0	0.14	0.63	9.43
9			48	0.21	0	0	0.08	0.72	13.15
9			72	0.29	0	0	0.03	0.73	14.40
10	0.20	1.00	24	0.24	0	0.02	0.14	0.75	10.32
10			48	0.25	0	0	0.08	0.78	14.12
10			72	0.31	0	0.01	0.04	0.80	14.31
11	0.40	0.00	24	0.26	0	0	0.16	0.56	8.00
11			48	0.31	0	0	0.10	0.64	12.04
11			72	0.27	0	0	0.04	0.67	13.77
12	0.40	0.20	24	0.22	0	0	0.14	0.62	9.24
12			48	0.29	0	0	0.09	0.69	13.55
12			72	0.28	0	0	0.03	0.70	14.01
13	0.40	0.40	24	0.25	0	0	0.15	0.69	10.15
13			48	0.29	0	0	0.09	0.75	13.64
13			72	0.33	0	0	0.05	0.77	14.40
14	0.40	0.60	24	0.24	0	0.02	0.14	0.73	10.89
14			48	0.31	0	0	0.09	0.79	13.84
14			72	0.34	0	0	0.04	0.78	14.19
15	0.40	1.00	24	0.26	0	0.02	0.13	0.76	11.35
15			48	0.32	0	0	0.08	0.82	14.30
15			72	0.29	0	0	0.05	0.83	14.54

At each level of M1 tested the addition of DISTILLASE® improved the fermentation rate and yield of ethanol, as shown in Figure 3, Panels A, B and C.

The starch analyses of the DDGS are shown in Table 4. From these analyses and the amount of DDGS, an estimate was then made of the amount of starch that remained unconverted in the fermenter. As indicated by these results, the addition of DISTILLASE® to M1 helps improve the hydrolysis of uncooked starch.

Table 4.

Trial	M1 GAU/g	Dist. GAU/g	% V/V Ethanol	DDGS gm DS	DDGS % Starch	% Unused Starch
1	0.10	0.00	6.95	13.93	52.37	46.75
2	0.10	0.20	11.44	8.88	26.84	15.29
3	0.10	0.40	12.98	7.29	15.60	7.29
4	0.10	0.60	13.63	6.74	9.53	4.12
5	0.10	1.00	14.14	6.68	5.60	2.40
6	0.20	0.00	11.33	9.51	33.64	20.51
7	0.20	0.20	13.09	7.53	16.84	8.13
8	0.20	0.40	13.65	6.72	8.88	3.83
9	0.20	0.60	14.40	6.37	3.75	1.53
10	0.20	1.00	14.31	6.36	2.50	1.02
11	0.40	0.00	13.77	7.00	9.43	4.23
12	0.40	0.20	14.01	6.78	8.57	3.73
13	0.40	0.40	14.40	6.38	3.12	1.28
14	0.40	0.60	14.19	6.44	2.02	0.83
15	0.40	1.00	14.54	6.41	1.61	0.66

Thus, the results obtained in these Examples indicate that adding a glucoamylase preparation with a GSH ratio greater than 1.5 to a glucoamylase with GSH ratio less than .6 can hydrolyze uncooked starch such that ethanol fermentations can be carried out on mashes that are not cooked. These results demonstrated the percent composition of a high GSH ratio glucoamylase to a low GSH ratio as low as 9% is very effective in hydrolyzing uncooked starch.

EXAMPLE 4

Influence of Stillage on the Fermentation of Cooked and Non-cooked Mash

This Example describes experiments designed to evaluate the fermentation of liquefied corn mash containing various levels of stillage compared to the fermentation of non-cooked corn mash containing various levels of stillage. The enzymes used in the fermentations were different. FERMENZYME® was used for fermenting the liquefied mash, which is a preparation that is similar to what is used commercially. For the non-cooked mash fermentation, a combination of DISTILLASE® and the RSH enzyme M1 were used.

The experiment was set up so that the corn solids would be constant while the solids from the stillage would vary. This meant that the total solids in the

fermenters increased as the stillage increased. Thin stillage was obtained from a local dry mill ethanol plant. The thin stillage was concentrated in a vacuum rotary evaporator to 44% solids. It was necessary to concentrate the thin stillage, so that the total solids in the fermenters would be manageable. The mash composition for each fermenter is shown in Table 5.

Table 5: Fermenter Mash Composition

31% Liquefied Corn at pH 5.0, .25 gm yeast,150 gm Total
.4 GAU/gm Fermenzyme

No	Liquefact gm	Stillage Syrup gm	Water gm	Mash % DS
1	130	0	20.0	31.1
2	130	2	18.0	31.7
3	130	5	15.0	32.5
4	130	10	10.0	34.0
5	130	15	5.0	35.5
6	130	20	0.0	36.9

31% Noncooked Corn <30 mesh at pH 5.0, .25 gm Yeast,150 gm Total
.6 GAU/gm Distillase + .19 RHU/gmM1 + GC106 equivalent

No	Corn gm	Stillage Syrup gm	Water gm	Mash % DS
7	52.4	0	97.1	30.9
8	52.4	2	95.1	31.5
9	52.4	5	92.1	32.4
10	52.4	10	87.1	33.9
11	52.4	15	82.1	35.3
12	52.4	20	77.1	36.8
13	52.4	25	72.1	38.3
14	52.4	30	67.1	39.7

The enzyme used for the liquefied corn mash was 0.4 GAU/gm of corn liquefact of FERMENZYME®. FERMENZYME® , a special blend of DISTILLASE® and a fungal protease for fermenting corn mash, is commercially available from Genencor®. For the non-cooked fermentations, a combination of DISTILLASE® and M1 were used along with the equivalent amount of protease that was in the FERMENZYME® used in the liquefied corn mash test runs. As indicated in Table 5, the solids in the fermenters varied (mash % DS). The results of the fermentations are given in Table 6, below.

Table 6: HPLC Profile During Fermentation

Ferm	Mash	Stillage gm	Enzyme	Hours	% W/V DP>2	% W/V DP-2	% W/V DP-1	% W/V Lactic	% W/V Glycerol	% V/V Ethanol
1	Liq	0	Fermenzyme	24	4.93	3.63	1.66	0.69	0.60	8.74
1				48	1.50	0.56	2.17	0.88	0.83	14.08
1				72	0.71	0.56	0.17	0.84	0.85	16.28
2	Liq	2	Fermenzyme	24	5.34	3.94	1.72	0.82	0.79	9.33
2				48	1.77	0.61	4.06	0.99	1.03	12.85
2				72	0.90	0.61	3.14	0.99	1.04	14.04
3	Liq	5	Fermenzyme	24	5.75	4.40	1.80	0.98	1.07	9.35
3				48	2.07	0.65	4.66	1.13	1.27	11.92
3				72	1.14	0.68	3.26	1.16	1.35	13.54
4	Liq	10	Fermenzyme	24	5.74	4.31	1.55	1.15	1.28	9.41
4				48	2.28	0.72	5.21	1.36	1.56	11.80
4				72	1.35	0.75	4.73	1.36	1.59	12.88
5	Liq	15	Fermenzyme	24	6.26	4.65	1.64	1.47	1.56	9.66
5				48	2.56	0.87	5.50	1.59	1.82	11.56
5				72	1.57	0.84	4.71	1.58	1.83	12.58
6	Liq	20	Fermenzyme	24	6.16	1.67	1.67	1.64	1.81	9.30
6				48	2.75	0.91	5.79	1.77	2.05	11.57
6				72	1.83	1.00	6.48	1.82	2.00	11.63
7	g. corn	0	Distillase + M1 + GC106	24	0.34	0	0.01	0.14	0.67	11.86
7				48	0.36	0	0	0.17	0.78	15.65
7				72	0.39	0	0	0.18	0.86	17.48
8	g. corn	2	Distillase + M1 + GC107	24	0.35	0	0.02	0.23	0.77	11.96
8				48	0.40	0	0	0.25	0.88	16.09
8				72	0.38	0	0	0.19	0.95	17.56
9	g. corn	5	Distillase + M1 + GC108	24	0.40	0	0.02	0.38	0.88	12.07
9				48	0.42	0	0	0.38	0.99	15.99
9				72	0.43	0	0	0.31	1.07	18.03
10	g. corn	10	Distillase + M1 + GC109	24	0.54	0	0.03	0.65	1.02	11.31
10				48	0.53	0	0	0.66	1.18	15.95
10				72	0.52	0	0	0.65	1.24	17.69
11	g. corn	15	Distillase + M1 + GC110	24	0.70	0	0	0.83	1.18	10.22
11				48	0.66	0	0.10	0.87	1.40	15.53
11				72	0.62	0	0	0.83	1.42	17.14
12	g. corn	20	Distillase + M1 + GC111	24	0.82	0	0	1.15	1.47	10.04
12				48	0.78	0	0	1.09	1.52	14.32
12				72	0.78	0	0	1.04	1.70	17.24
13	g. corn	25	Distillase + M1 + GC112	24	1.04	0	0	1.48	1.78	7.71
13				48	1.01	0	0	1.40	1.77	11.40
13				72	0.99	0	0	1.42	1.97	14.72
14	g. corn	30	Distillase + M1 + GC113	24	1.17	0	0	1.74	2.02	7.68
14				48	1.18	0	0	1.70	2.06	11.61
14				72	1.12	0	0	1.65	2.18	14.58

In commercial practice, a certain amount of the stillage is recycled for the yeast nutrient content and to help the water balance in the plant. Thus, fermentation systems that are less influenced by stillage is very desirable in industrial fermentation plants. The results of these experiments show that stillage effects the fermentation of liquefied mash more than in non-cooked mash.

Figure 4 shows the response of ethanol to the amount of stillage added in both types of mashes. In both cases, increasing the stillage solids reduced the ethanol level. But as Figure 4 shows, the non-cooked mash is much less sensitive to stillage than the cooked mash.

Figure 5 shows the glucose profile after 72 hour of fermentation and the results are very striking. As the stillage solids increased in cooked mash more glucose was left unfermented, while in the non-cooked mash, essentially non-detectable levels of glucose were observed at all levels of stillage. This observation is very significant because as the starch was hydrolyzed it was immediately to ethanol by the yeast. This level of glucose build-up is very unusual. This observation is also important particularly with respect to the yeast, in that even though the glucose level is extremely low, the yeast remain very active in fermenting. In contrast, in the cooked mash, even when glucose was in ample supply, the yeast could not ferment the glucose.

Figure 6 is a plot of the disaccharides after 72 hours of fermentation with respect to stillage added. The disaccharide levels for the non-cooked mash were found to be essentially non-detectable throughout the range of stillage added, but in the cooked mash as the stillage level increased the disaccharides increased.

As indicated in Figure 7, the higher sugars (*i.e.*, oligosaccharides greater than disaccharides) provided a somewhat a similar picture, as the level of higher sugars for the cooked mash were higher with respect to stillage added than for the non-cooked mash.

Figure 8 shows the lactic acid level after 72 hours of fermentation. As indicated, the levels are higher for the cooked mash than the non-cooked mash. One consideration with lactic acid is that it is a measure of contamination. Although it is not intended that the present invention be limited to any particular theory or mechanism, it is possible that since both the glucose and disaccharide levels are always very low in the non-cooked mash, contaminating microorganisms have very little substrate to utilize.

Figure 9 provides a summary of the glycerol levels after 72 hour of fermentation. As indicated, again the levels are lower at the respective stillage addition levels with the non-cooked mash than with the cooked mash. A

contributing factor for glycerol formation during fermentation with yeast is the stress the yeast is under. Generally, the more stress the yeast is under, the more glycerol that will be formed. The results in Figure 9 would indicate that at similar stillage levels, the yeast in the non-cooked mash are under less stress. But even at higher stillage levels that could be run with cooked mash, higher levels of glycerol were formed. Even with the higher glycerol level in the non-cooked mash, the yeast produced more ethanol. Thus, it appears that yeast seem to ferment more efficiently in the non-cooked mash than in the cooked mash.

Various other examples and modifications of the foregoing description and examples will be apparent to a person skilled in the art after reading the disclosure without departing from the spirit and scope of the invention, and it is intended that all such examples or modifications be included within the scope of the appended claims. All publications and patents referenced herein are hereby incorporated by reference in their entirety.

CLAIMS

1. A method for producing an alcohol as an end-product comprising the steps of:

a) contacting a carbon substrate and at least one substrate-converting enzyme to produce an intermediate; and

b) contacting said intermediate with at least one intermediate-converting enzyme, wherein said intermediate is substantially all converted by said intermediate enzyme to said alcohol.

2. The method of Claim 1, wherein said intermediate-converting enzyme is a microbial enzyme.

3. The method of Claim 2, wherein said intermediate-converting microbial enzyme is secreted by a microorganism in contact with said intermediate.

4. The method of Claim 1, wherein said substrate-converting enzyme is a microbial enzyme.

5. The method of Claim 4, wherein said substrate-converting microbial enzyme is secreted by a microorganism in contact with said substrate.

6. The method of Claim 1, wherein said intermediate-converting enzyme and said substrate-converting enzyme are produced by microorganisms of the same species.

7. The method of Claim 1, wherein said intermediate-converting enzyme and said substrate-converting enzyme are produced by microorganisms of the different species.

8. The method of Claim 1, wherein concentration level of said intermediate is maintained at a level below that which triggers catabolite repression effects upon the conversion of said intermediate to said end-product.

9. The method of Claim 1, wherein concentration level of said intermediate is maintained at a level below that which triggers enzymatic inhibition effects upon the conversion of said intermediate to said end-product.

10. The method of Claim 1, wherein said intermediate is converted to said end-product at a rate sufficient to maintain the concentration of said at less than 0.25%.

11. The method of Claim 1, wherein said substrate is selected from the group consisting of biomass and starch.

12. The method of Claim 11, wherein said biomass comprises corn solids.

13. The method of Claim 1, wherein said intermediate is selected from the group consisting of hexoses and pentoses.

14. The method of Claim 13, wherein said hexose is glucose.

15. The method of Claim 1, wherein said contacting said substrate and substrate-converting enzyme further comprises bioconverting said substrate to produce said intermediate.

16. The method of Claim 1, wherein said alcohol end-product is ethanol.

17. The method of Claim 1, wherein the step of contacting said substrate and said at least one substrate-converting enzyme further comprises providing an amount of said substrate-converting enzyme at a concentration that produces said

intermediate at a concentration that is less than or equal to the amount of said intermediate converted by said at least one intermediate-converting enzyme.

18. The method of Claim 1, wherein said at least one substrate-converting enzyme converts at least 50% of said substrate to said intermediate within 72 hours.

19. The method of Claim 18, wherein said at least one substrate-converting enzyme converts at least 90% of said substrate to said intermediate within 72 hours.

20. The method of Claim 19, wherein said at least one substrate-converting enzyme converts at least 95% of said substrate to said intermediate within 72 hours.

21. The method of Claim 1, wherein said at least one substrate-converting enzyme and said at least one intermediate-converting enzyme are obtained from a microorganism selected from the group consisting of *Rhizopus* and *Aspergillus*.

22. A method for producing alcohol as an end-product comprising the steps of:

a) contacting a carbon substrate and at least one substrate-converting enzyme to produce an intermediate; and

b) contacting said intermediate with at least one intermediate-converting enzyme, wherein said intermediate is substantially all converted by said intermediate enzyme to said alcohol end-product, and wherein the presence of said end-product does not inhibit the further production of said alcohol end-product.

23. The method of Claim 22, wherein said intermediate-converting enzyme is a microbial enzyme.

24. The method of Claim 22, wherein said intermediate-converting microbial enzyme is secreted by a microorganism in contact with said intermediate.

25. The method of Claim 22, wherein said substrate-converting enzyme is a microbial enzyme.

26. The method of Claim 22, wherein said substrate-converting microbial enzyme is secreted by a microorganism in contact with said substrate.

27. The method of Claim 22, wherein said intermediate-converting enzyme and said substrate-converting enzyme are produced by microorganisms of the same species.

28. The method of Claim 22, wherein said intermediate-converting enzyme and said substrate-converting enzyme are produced by microorganisms of the different species.

29. The method of Claim 22, wherein said alcohol end-product is ethanol.

30. A method for producing an alcohol end-product comprising the steps of:

a) contacting a carbon substrate and at least one substrate-converting enzyme to produce an intermediate; and

b) contacting said intermediate with at least one intermediate-converting enzyme, wherein said intermediate is substantially all converted by said intermediate enzyme to said alcohol end-product, and wherein the presence of said substrate does not inhibit the further production of said alcohol end-product.

31. The method of Claim 30, wherein said intermediate-converting enzyme is a microbial enzyme.

32. The method of Claim 30, wherein said intermediate-converting microbial enzyme is secreted by a microorganism in contact with said intermediate.

33. The method of Claim 30, wherein said substrate-converting enzyme is a microbial enzyme.

34. The method of Claim 30, wherein said substrate-converting microbial enzyme is produced is secreted by a microorganism in contact with said substrate.

35. The method of Claim 30, wherein said intermediate-converting enzyme and said substrate-converting enzyme are produced by microorganisms of the same species.

36. The method of Claim 30, wherein said intermediate-converting enzyme and said substrate-converting enzyme are produced by microorganisms of the different species.

37. The method of Claim 36, wherein said alcohol end-product is ethanol.

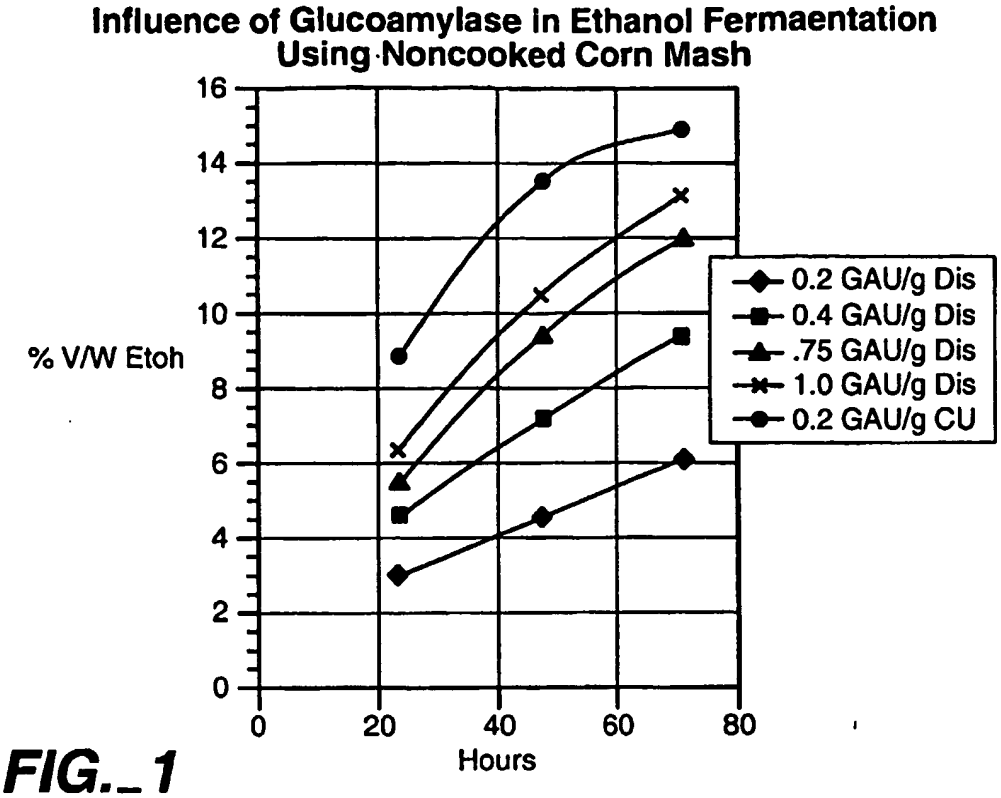
Influence of Glucoamylase in Ethanol Fermaentation
Using Noncooked Corn Mash
% V/W Etoh
16
14
12
10
8
6
4
2
0
0
20
40
60
80
Hours
0.2 GAU/g Dis
0.4 GAU/g Dis
.75 GAU/g Dis
1.0 GAU/g Dis
0.2 GAU/g CU

FIG._1

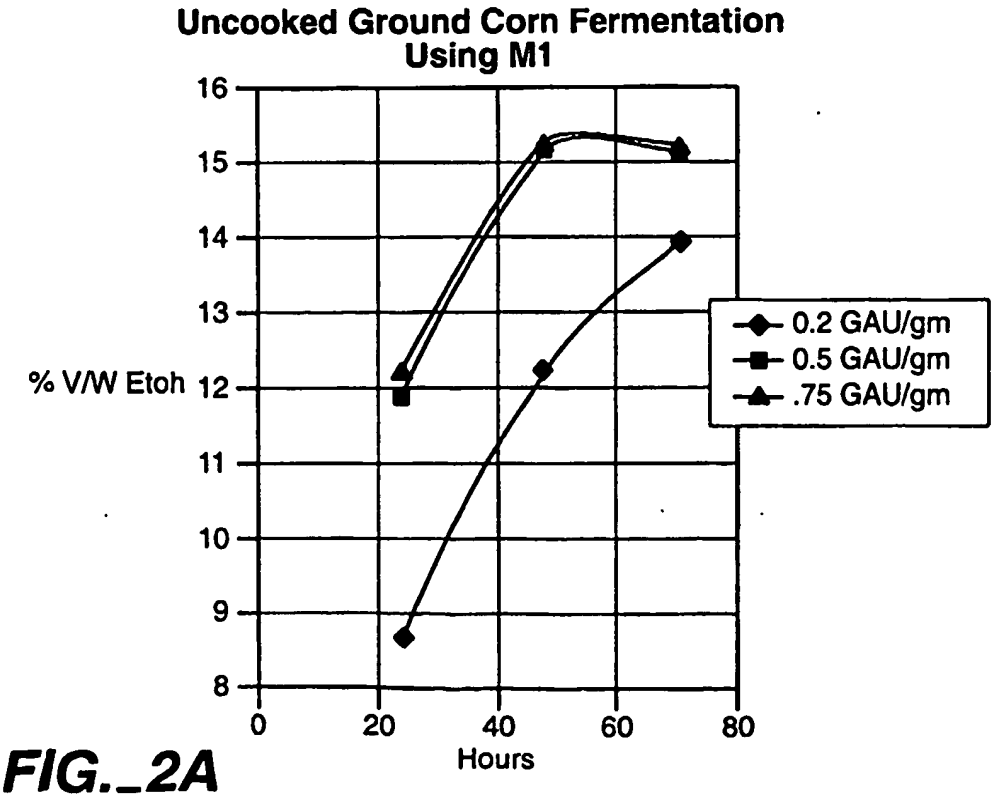
Uncooked Ground Corn Fermentation
Using M1
% V/W Etoh
16
15
14
13
12
11
10
9
8
0
20
40
60
80
Hours
0.2 GAU/gm
0.5 GAU/gm
.75 GAU/gm

FIG._2A

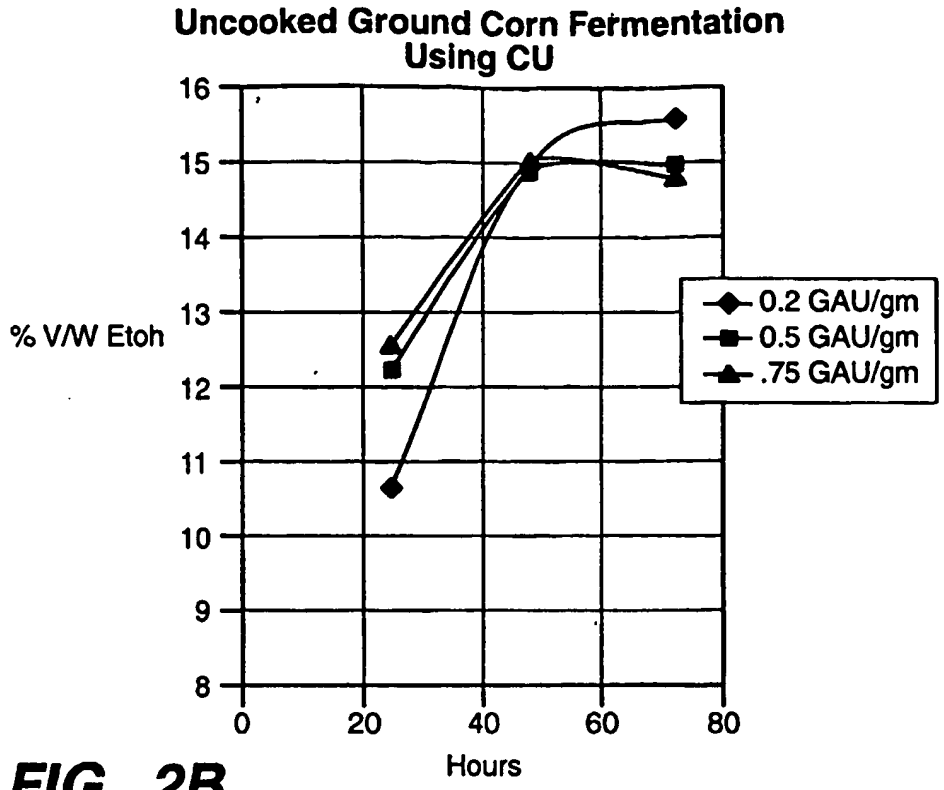
Uncooked Ground Corn Fermentation
Using CU
% V/W Etoh
16
15
14
13
12
11
10
9
8
0
20
40
60
80
Hours
0.2 GAU/gm
0.5 GAU/gm
.75 GAU/gm

FIG._2B

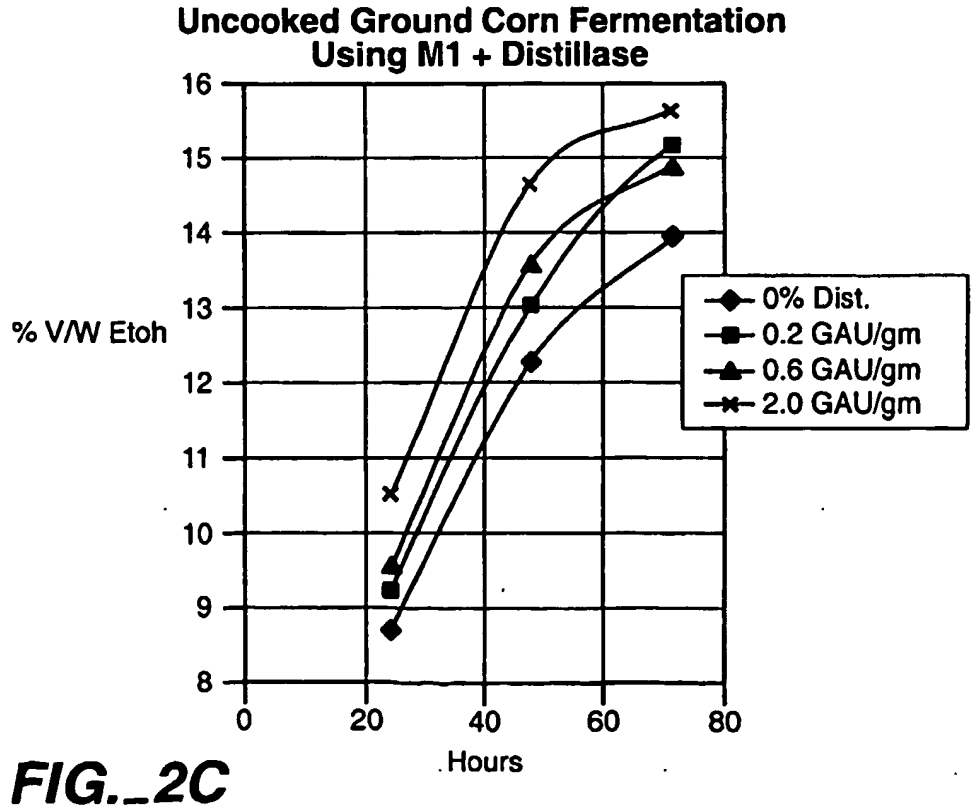
Uncooked Ground Corn Fermentation
Using M1 + Distillase
% V/W Etoh
16
15
14
13
12
11
10
9
8
0
20
40
60
80
Hours
0% Dist.
0.2 GAU/gm
0.6 GAU/gm
2.0 GAU/gm

FIG._2C

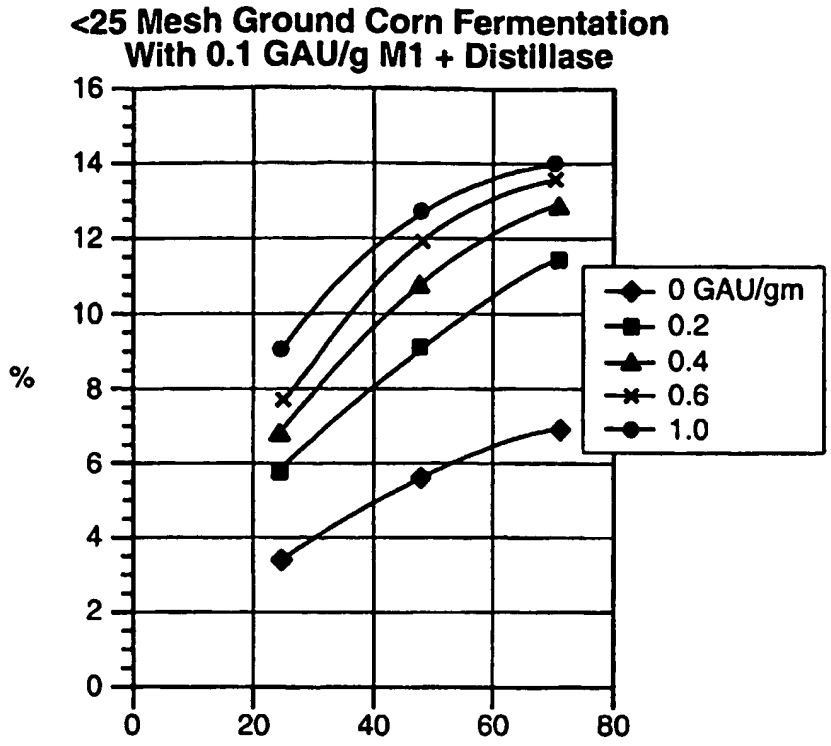
<25 Mesh Ground Corn Fermentation
With 0.1 GAU/g M1 + Distillase
%
Hours
0 GAU/gm
0.2
0.4
0.6
1.0
0
2
4
6
8
10
12
14
16
0
20
40
60
80

FIG._3A

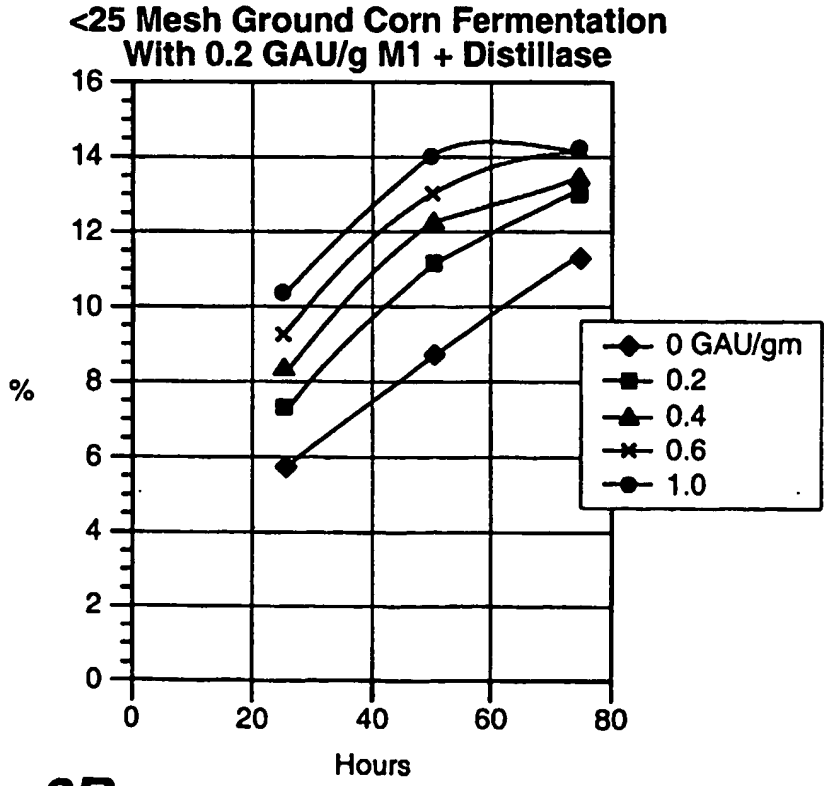
<25 Mesh Ground Corn Fermentation
With 0.2 GAU/g M1 + Distillase
%
Hours
0 GAU/gm
0.2
0.4
0.6
1.0
0
2
4
6
8
10
12
14
16
0
20
40
60
80

FIG._3B

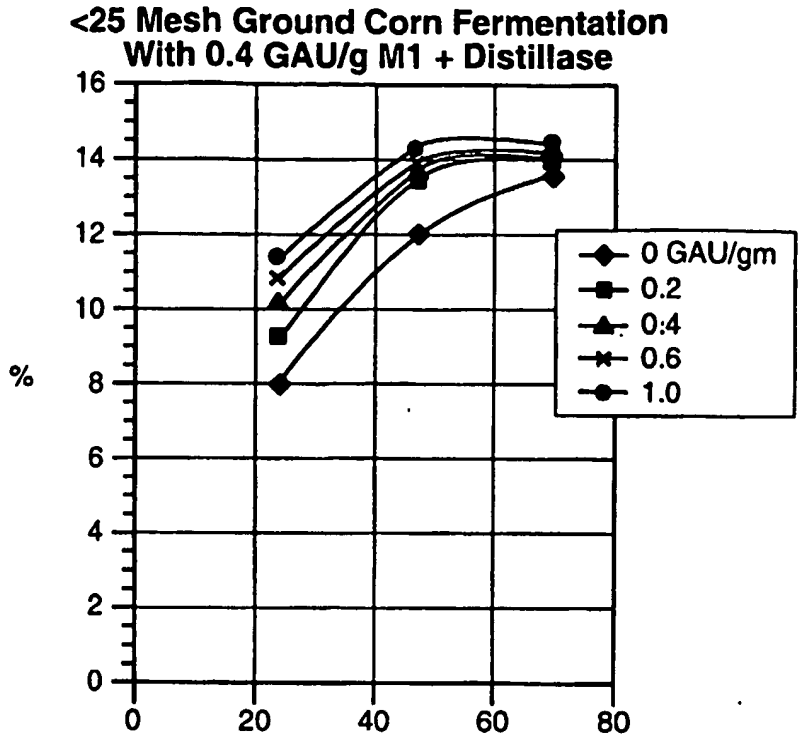
<25 Mesh Ground Corn Fermentation
With 0.4 GAU/g M1 + Distillase
%
Hours
0 GAU/gm
0.2
0:4
0.6
1.0
0
2
4
6
8
10
12
14
16
0
20
40
60
80

FIG._3C

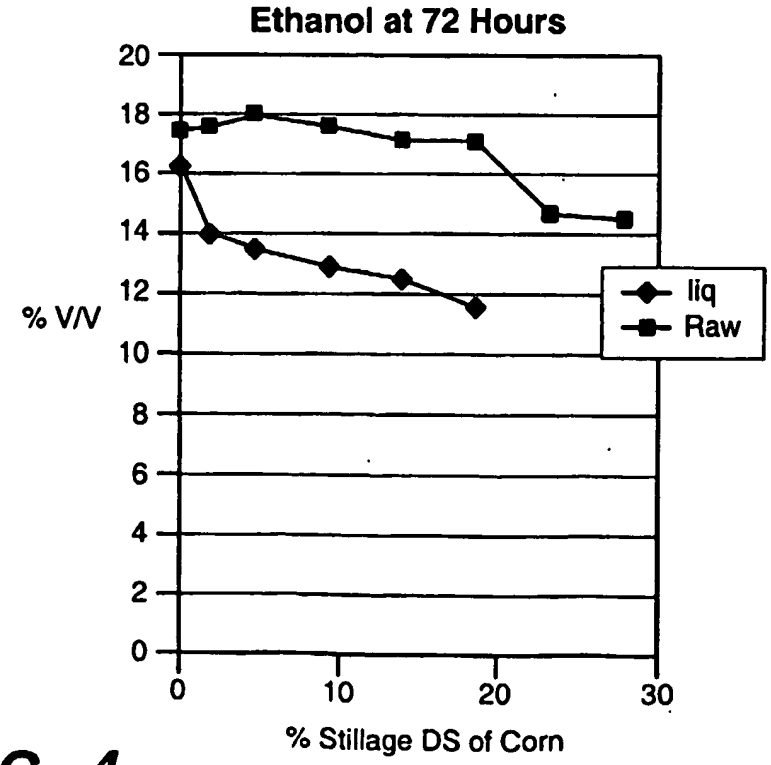
Ethanol at 72 Hours
% V/V
% Stillage DS of Corn
liq
Raw
0
2
4
6
8
10
12
14
16
18
20
0
10
20
30

FIG._4

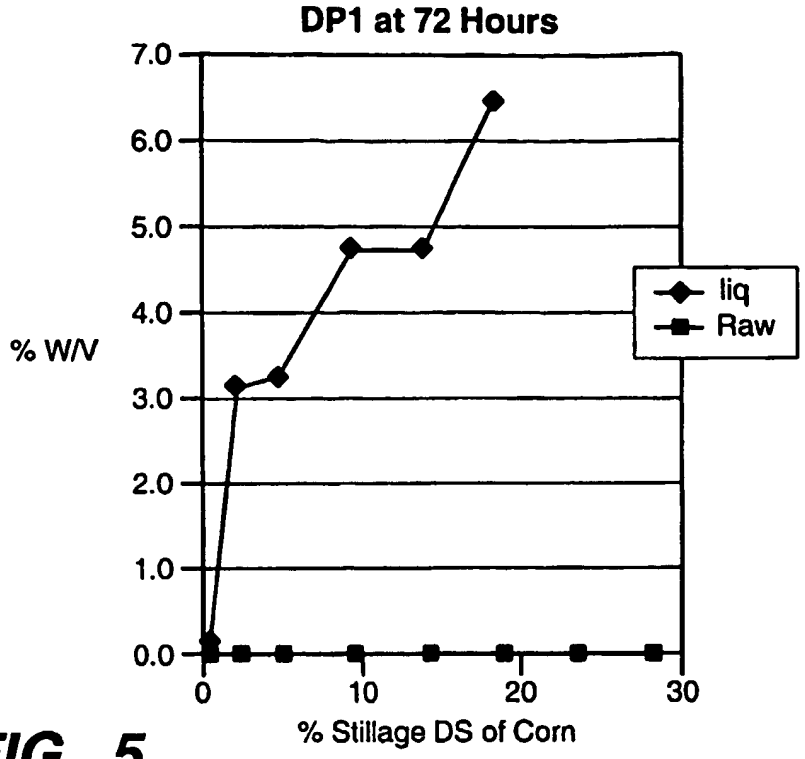
DP1 at 72 Hours
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
% W/V
liq
Raw
0
10
20
30
% Stillage DS of Corn

FIG._5

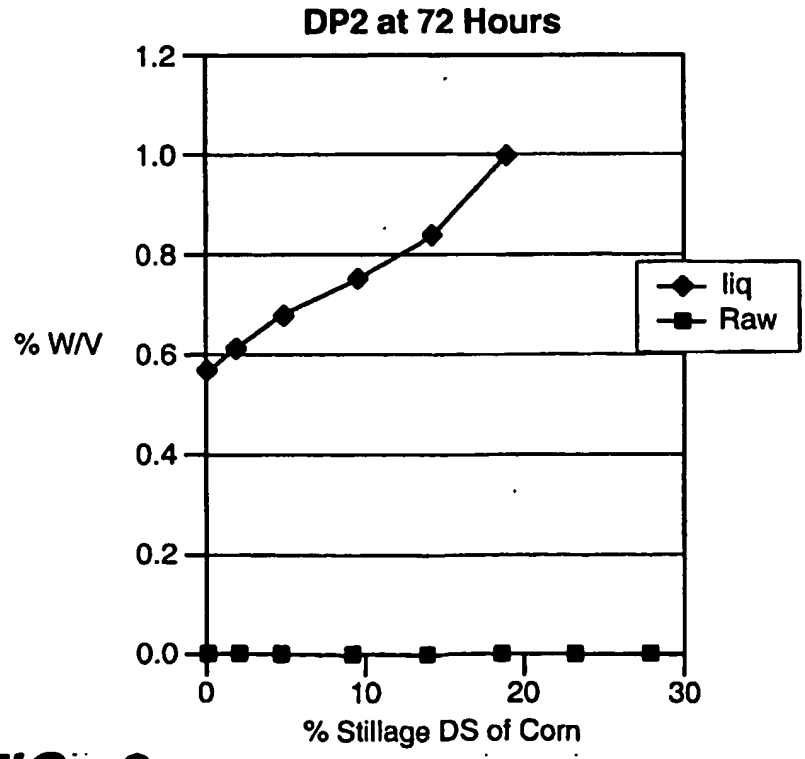
DP2 at 72 Hours
1.2
1.0
0.8
0.6
0.4
0.2
0.0
% W/V
liq
Raw
0
10
20
30
% Stillage DS of Corn

FIG._6

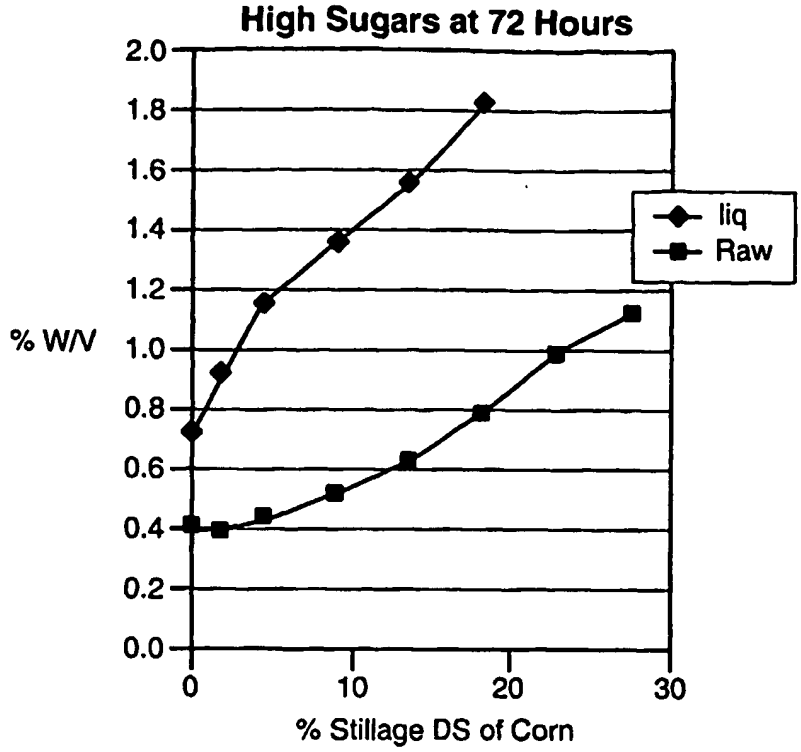
High Sugars at 72 Hours
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
% W/V
0
10
20
30
% Stillage DS of Corn
liq
Raw

FIG._7

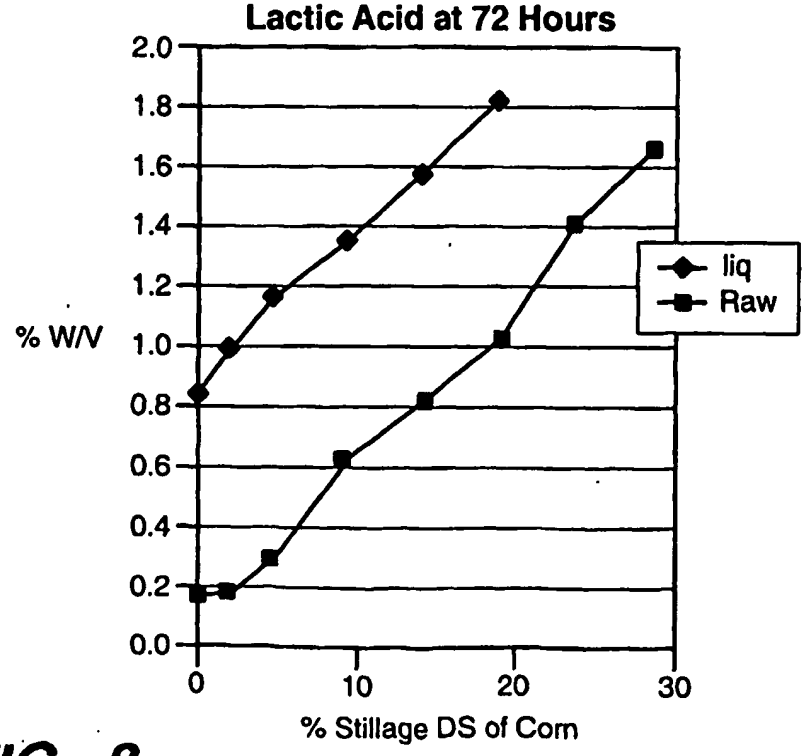
Lactic Acid at 72 Hours
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
% W/V
0
10
20
30
% Stillage DS of Corn
liq
Raw

FIG._8

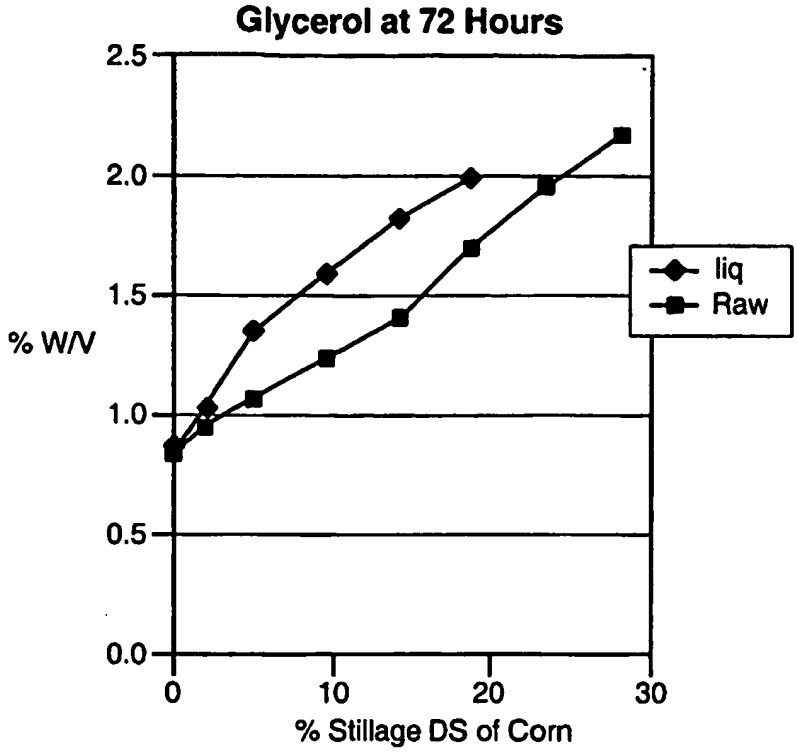
Glycerol at 72 Hours
2.5
2.0
1.5
1.0
0.5
0.0
% W/V
0
10
20
30
% Stillage DS of Corn
liq
Raw

FIG._9